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ELECTRICAL ENERGY OUTPUT OF THE RESTING STOMACH AS DETERMINED BY SHUNTING ITS POTENTIAL

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Although many investigators have suggested, partly on the basis of the results of experiments on non-living membranes and partly on the basis of potential measurements in living tissues, that electroendosmosis may be the mechanism by which osmotic work is performed by living organisms (1, 5, 6), the actual experimental evidence for this hypothesis is extremely meager. This hypothesis implies living tissues are capable of producing sufficient electrical energy for the performance of their osmotic work. A review of the literature reveals that there have been comparatively few studies on the production of electrical energy by living organisms. However, Stapp (11) has attempted to determine the ability of the frog skin to produce electrical energy by shunting its potential through a relatively low external resistance. He concluded that the electrical energy produced under the conditions of his experiments was in the neighborhood of 1 to 5 per cent of the total metabolic energy. Blinks (2) has reported the production through an external shunt of a continuous current of from 5 to 10 microamperes from a single halycystis cell.

For an accurate determination of the amount of electrical energy produced by an organism the actual electrical circuits inside the tissue would have to be known. Since these internal circuits are not known it has to be assumed for the purposes of calculating the electrical energy that the circuit of the tissue consists of a potential and a resistance in series. Calculations based on this assumption would therefore represent only the minimum possible output of electrical energy (see discussion). Since the method of shunting the current through an external resistance is limited to determining the minimum possible output of electrical energy of a tissue, it would be interesting to know whether or not there is any depolarization of the potential upon the withdrawal of the current. In other words, to what extent is the ability of a tissue to maintain its potential taxed by the withdrawal of the maximum current. The present work is primarily concerned with this problem. It would also be of interest to determine by this method the electrical energy output of a tissue capable of doing considerable osmotic work. The dog's stomach was chosen because it is a

structure capable of producing considerable osmotic work in the secretion of HCl (4) and also because reports in the literature and preliminary experiments revealed that it possessed a relatively stable electrical potential which would be advantageous for the study of effects of current flow on the magnitude of the potential. Several workers have investigated the effect on the gastric potential of drugs that stimulated gastric secretion. Quigley et al. (9) report that these agents have no observable effect on the potential while Sarre (10) and Mislowitzer (7) report a decrease in the stomach potential after histamine stimulation. In preliminary experiments of the present investigation a decrease in the magnitude of the stomach potential was observed after histamine stimulation. Since the method of shunting cannot give information about the actual electrical energy expended inside a tissue, and since the magnitude of the potential was higher in the resting stomach than after histamine stimulation, it was decided to use the resting stomach as the material for this investigation.

Methods. Dogs starved for 24 hours after preliminary ether anesthesia were injected with either barbital (200 to 250 mgm. per kgm.) or pernoston[1] (35 to 60 mgm. per kgm.). The stomach was exposed through an abdominal incision, ligatures were placed around the lower part of the esophagus and the pylorus, and an incision was made in the ventral wall of the stomach through which an electrode could be introduced. The stomach was washed out with large quantities of warm physiological saline. Under these conditions there was practically no spontaneous secretion, i.e., less than 1 cc. per hour.

An electrode introduced through the incision in the ventral wall was placed on the mucosal side on the ventral wall of the body of the stomach. Another similar electrode was placed on the serosal side directly opposite the inside electrode. An electrode consisted essentially of a circular shell of lucite containing a zinc plate which had previously been immersed in a saturated zinc amalgam, surrounded by saturated zinc acetate agar (2 per cent) with an outer layer of mammalian Ringer agar (2 per cent) to prevent zinc acetate from coming in contact with the stomach. Several other types of electrodes were tried but none were as satisfactory as the ones described above. Ag-AgCl electrodes showed considerable polarization with the current strengths of the same order of magnitude as those obtained from the stomach.

In order to prevent the electrodes from exerting undue pressure on the stomach one of the electrodes was fixed and the second electrode was free to swing. The support of the second electrode was placed so that the stomach supported a given fraction of the weight of this electrode. The actual pressure exerted by the electrode was less than 4 cm. of water.

These electrodes possessed a potential difference that was usually only a small fraction of a millivolt and showed very little polarization, i.e., usually less than $\frac{1}{2}$ millivolt change with a current density of $\frac{1}{2}$ milliampere per $cm.^2$ for a period of five minutes. Several sizes of electrodes were used, all of relatively large surface area to minimize the contribution of neighboring areas, not directly under the electrodes, to the production of current.

[1] The Riedel-deHaen Co., New York, kindly supplied the pernoston used in these experiments.

In preliminary experiments with thin layers of Ringer agar a white deposit appeared on the stomach in a relatively short time. It was found necessary to use thicker layers of Ringer agar, i.e., about 1 cm. for each electrode. The thicker layers of Ringer agar increased the resistance of the electrodes, the average resistance of the electrodes (two electrodes in series) used in the experiments reported here being 16.4 ohms per 10 $cm.^2$ of electrode area.

Leads from the electrodes were connected to a variable resistance box. The voltage drop across the resistance box was measured with either a potentiometer or a galvanometer. The resistance of the electrodes plus stomach was found to be relatively low and for the later experiments the galvanometer, calibrated as a voltmeter, was used in place of the potentiometer. The leads from the resistance box, for measuring the voltage drop across the box or directly from the electrodes, for open circuit voltage measurements, were connected to a resistance of approximately 26,000 ohms and the galvanometer leads were connected across portions of this resistance (100 ohms for open circuit voltage measurements) depending upon the sensitivity desired. With this arrangement wide variations in the resistance of the stomach and electrodes would have a negligible effect on the galvanometer deflection. The error in using this method for measurement of the open circuit voltage (referred to as potential in this paper) or the voltage drop across the resistance box was less than 0.2 per cent. Current flow through the resistance box was calculated from the determinations of the voltage drops. A direct current Wheatstone bridge was used for measuring the resistance of the electrodes. Since the potential difference of the electrodes was never actually zero the resistance was determined by measuring the apparent resistance in the two directions and averaging these values.

Results. In the early experiments it was found that the potential of the stomach tended to decrease during the course of an experiment. However, in the later experiments in which precautions were taken to maintain the temperature of the stomach at a normal level, and in which a minimum of the anesthetic was used the potential was maintained at a relatively constant value for the duration of an experiment. The serosa was found to be positive in the external circuit to the mucosa in every experiment. The magnitude of the potentials was similar to those found by other investigators (7, 9, 12).

Effect of decreasing external resistance on current flow. In figure 1A, a typical experiment, the current produced by the stomach is plotted against the total external resistance of the circuit. The external resistance was regulated by varying the resistance of the variable resistance box, the total external resistance being equal to the resistance of the resistance box plus the resistance of the electrodes. The lowest external resistance was equal to the resistance of the electrodes plus 1 ohm. Over 40 similar experiments were performed and similar results were obtained in all of them. As is shown in the figure, decreasing the external resistance increases the current, the rate of increase being greatest when the resistance is less than 100 ohms.

Effect of current flow on the magnitude of the potential. An answer to the question as to whether there is depolarization of the potential upon the withdrawal of current may be obtained by applying the following well known principle of

physics. If a potential difference exists between two points in a circuit, a potential and resistance in series can represent the circuit for predictions of the current in shunts connecting the two points. That is, if there are two terminals on a closed box and nothing is known about the circuit inside the box, a resistance and potential of appropriate magnitudes arranged in series constitute an equivalent circuit for the actual circuit inside the box with respect to predictions of the amount of current flowing through shunts connecting the terminals of the box. This is true providing of course that the flow of current in itself does not cause a reduction in the magnitude of the potentials in the circuit. Applying this principle to the stomach it follows from Ohm's law that

$$E = R_E I + R_S I \tag{1}$$

where E is the potential of the stomach, R_S the resistance of the stomach, R_E the resistance in the external circuit, and I the current in the external circuit. On rearranging

$$R_E I = E - R_S I \tag{2}$$

From equation 2 it can be seen that $R_E I$ vs. I can be represented by a straight line providing there is no decrease in E as the magnitude of the current increases. E is equal to the total IR drop in the circuit. From the data presented above (R_E vs. I) $R_E I$ was calculated and plotted against I. In figure 1A it can be seen that a straight line accurately represents this relationship. This straight line relationship was found in all of the experiments except in a few early experiments in which measurements were attempted during periods in which the potential of the stomach was not very stable. Inspection of equation 2 reveals that the slope of the line is numerically equal to the resistance of the stomach. The resistance of the stomachs in these experiments is given in column R_S, table 1. The resistance of the stomach per square centimeter can be obtained by multiplying R_S by the electrode area. The resistances were also determined by means of a direct current Wheatstone bridge. The values obtained by the two methods were the same within the limits of error of the methods. The measurements obtained by the Wheatstone bridge method are not given here because they are not essential to the thesis presented in this paper.

Effect of shunting for various periods of time on the current output. In the above experiments the electrodes and stomach were connected to the variable resistance box just long enough to obtain a reading. The time involved for a complete series of readings was about three minutes. The question naturally arises as to whether the original output of current can be maintained over longer periods of time. Figure 1B represents a typical experiment in which the current was shunted through the electrodes plus a 1 ohm resistor for a period of five minutes. It can be seen from the graph that apart from a small temporary initial decrease in the current, the current output remained at a relatively constant level. Over 30 similar experiments were performed in which the current was shunted through the electrodes plus a 1 ohm resistor for periods of a few minutes to $\frac{1}{2}$ hour. In all of the experiments in which the open circuit voltage was relatively constant

before shunting (the majority of experiments), the current, during the period the shunt was applied, was maintained at approximately its initial value. It showed no tendency to decrease with time. In the experiments in which the open circuit voltage was either gradually decreasing or increasing, the voltage drop through the 1 ohm resistor showed the same proportionate tendency to change as did the open circuit voltage before shunting. Interpolation of the open circuit

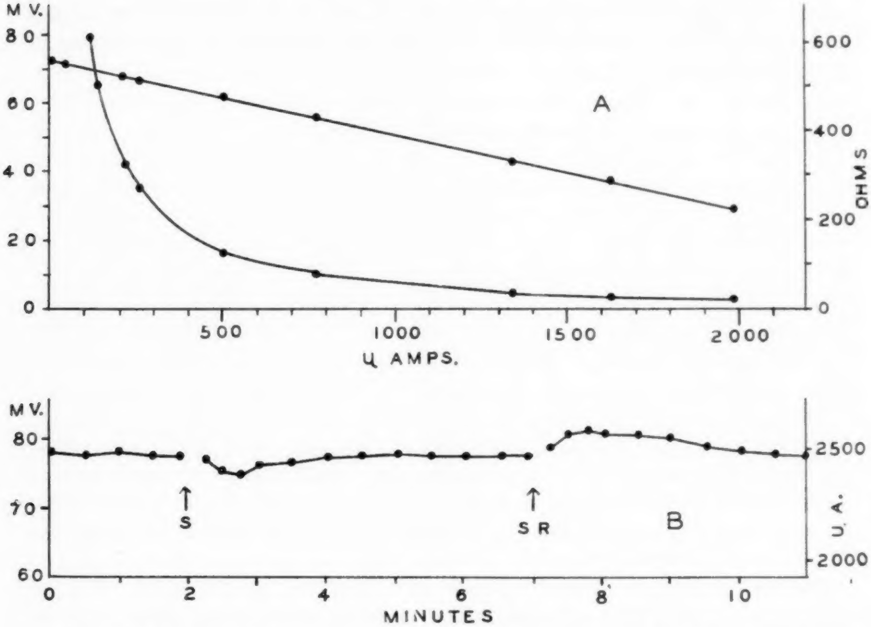

Fig. 1A. The curved line represents the current in the external circuit in microamperes vs. resistance of the external circuit (resistance of the electrodes plus resistance of variable resistance box) in ohms. The lowest external resistance in this experiment was 16.5 ohms (resistance of the electrodes, 15.5 ohms plus 1 ohm).

The straight line represents the IR drop in the external circuit in millivolts (R_EI) vs. the current in the external circuit. Dog 3 of table 1.

Fig. 1B. Effect of shunting the stomach through the electrodes plus a 1 ohm resistor. The line before *S* and after *SR* represents the open circuit voltage of the stomach in millivolts (left-hand scale). At *S* the stomach was shunted; at *SR* the shunt was removed. The line between *S* and *SR* represents the current in the external circuit in microamperes (right-hand scale) during the period in which the shunt was applied. Dog 10 of table 1.

voltages before and after shunting indicated that shunting did not observably influence the rate of change of the potential.

It can be seen in figure 1B that after the shunt was removed there was a small temporary increase in the potential. The two effects, the small temporary decrease in the current after applying the shunt and the small temporary increase in the potential after removal of the shunt, were observed in the majority of

the experiments in which the magnitude of the current exceeded 150 microamperes per square cm. It was never more than 3 per cent of the average values of the current or potential. Controls demonstrated that these effects could not be due to peculiarities of the galvanometer used for measuring the potentials. Current from a comparable potential and resistance when connected to the galvanometer showed only a difference of $\frac{1}{4}$ mv. between the initial 15 second reading and the subsequent readings. Controls in which comparable currents were passed through the electrodes showed a maximum change in P.D. of less than $\frac{1}{2}$ mv. These effects seem therefore to be mainly dependent on the activity of the stomach.

TABLE 1

	DOG									
	1	2	3	4	5	6	7	8	9	10
R_S	11.1	7.5	21.0	12.1	24.5	20.0	24.4	11.2	10.2	12.1
Elec. area	13.8	21.3	13.8	11.6	11.6	11.6	11.6	10.7	10.7	10.7
I_{max}	938	3,040	2,040	1,210	1,520	1,170	1,340	2,950	1,830	2,580
$\frac{I_{max}}{\text{cm.}^2}$	68	142	150	104	131	101	115	276	171	241
E	27.6	47.1	71.5	29.1	55.5	41.2	51.5	80.5	44.4	80.8
$\frac{EI}{\text{cm.}^2}$	1.88	6.74	10.7	3.0	7.3	4.2	5.9	22.2	7.6	19.4

R_S is the resistance of the stomach in ohms as determined from equation 2. "Elec. area" is the area of electrodes in square centimeters. $I_{max.}$ is the current in microamperes obtained when external resistances equal the resistance of the electrodes plus 1 ohm. $\frac{I_{max.}}{\text{cm.}^2}$ is the current in microamperes $I_{max.}$ divided by the electrode area. E is the average of the potentials in millivolts before and after $I_{max.}$ was obtained. $\frac{EI}{\text{cm.}^2}$ is the rate of electrical energy production in microwatts per square centimeter obtained by multiplying E by $\frac{I_{max.}}{\text{cm.}^2}$. Pernoston was used in the third and last four experiments; barbital was used in the other experiments.

The maximum current obtained for each of the dogs is given in column $I_{max.}$ of table 1. The area of the electrode used in each of the experiments is also given in table 1. The maximum current per square centimeter was calculated by dividing the maximum current by the electrode area.

Calculation of electrical energy output of the stomach. Since current can be drawn from the stomach over comparatively long periods of time and since there is no maintained depolarization of the potential during the flow of current, the rate of electrical energy production under these conditions can be calculated by multiplying the current by the open circuit voltage. In table 1 column $\frac{\text{EI}}{\text{cm.}^2}$ gives the rate of production of electrical energy in microwatts per sq. cm. This was calculated from the average of the open circuit voltage before and after shunting

(column E) and the maximum current output (column $\frac{I_{max.}}{cm.^2}$) per cm.^{2}. The average value for electrical energy output was 8.9 microwatts per sq. cm.

Discussion and conclusions. The foregoing experiments demonstrate that as much as 67.8 to 276 microamperes per cm.^{2} can be produced continuously by the resting stomach without any appreciable maintained depolarization of the potential. Had there been marked depolarization of the potential it would indicate that these amounts of current would be near the maximum that the potential was capable of producing. The fact that there was no appreciable depolarization suggests that the gastric potential is inherently capable of delivering currents of greater magnitudes than those actually obtained. It is to be recalled that the calculations of the electrical energy produced by the stomach were made on the assumption that a potential and resistance represented the actual circuit. It is obvious that if any other circuit better approximated the actual circuit of the stomach, the amount of electrical energy produced would be greater than that calculated. For example, if there were a shunt across the potential inside the wall of the stomach and the resistance of the shunt was small compared to the resistance of the tissues from the locus of the potential to the serosal or mucosal surfaces then the amount of electrical energy might be many times greater than that calculated. It should be pointed out here with respect to the problem of determining the actual circuit of the stomach that, since a resistance and a potential in series constitute an equivalent circuit for the stomach, the sort of data presented in this paper will not enable one to determine the actual circuit of a tissue. A resistance and potential is an equivalent circuit for the data presented in this paper and it is not to be assumed that it is an equivalent circuit for all of the electrical characteristics of the stomach.

Inspection of figure 1A in which current is plotted against resistance might suggest that as the external resistance approaches zero the current increases to infinity and therefore the problem of determining the ability of the stomach to produce electrical energy is primarily one of obtaining lower values of the external resistance. That this is not the case can be shown by an examination of the equation representing the above relationship, i.e.,

$$I = \frac{E}{R_E + R_S} \tag{3}$$

obtained from equation (1). It is obvious that as R_E approaches zero I approaches $\frac{E}{R_S}$.

In order to obtain an approximate idea of the relative order of magnitude of the electrical energy output a comparison of these values with the values found in the literature for the amount of metabolic energy produced by the resting stomach would be pertinent. Ni and Lim (8) give 0.004 cc. of O_2 per gram per minute as an average value of the O_2 consumption of the resting vivi-perfused stomach. Boenheim (3) gives 166 mm.^{3} of O_2 per 100 grams per minute. For purposes of comparison the metabolic energy was calculated in electrical units.

The above figures in microwatts per sq. cm. using Ni and Lim's data for conversion from grams to sq. cm. are 472 and 200 respectively. Using the average value for the electrical energy obtained in these experiments, 8.9 microwatts per sq. cm. (average of the values in column $\frac{E_1}{\text{cm.}^2}$ of table 1), the per cent of total metabolic energy obtained as electrical energy is 1.9 per cent and 4.4 per cent, which is approximately the same percentage that Stapp (11) found for the frog skin. The actual amount of electrical energy production found by Stapp for the frog skin was about one-tenth of that found for the stomach.

The question then arises: Is there enough electrical energy produced by the stomach to account for the osmotic work performed in the secretion of gastric juice? Calculations reveal that the amount of electrical energy per stomach per 24 hours, using the maximum rate of electrical energy production (22.2 microwatts per sq. cm.), is equivalent to the minimum free energy necessary to produce approximately 200 ml. of gastric juice (using Davenport's (4) value of 820 cal. per liter). Although the stomach is capable of producing more gastric juice than the above amount, it is nevertheless of the same order of magnitude as the amounts actually produced. The fact that there was no maintained depolarization of the potential during the flow of the maximum current suggests that the stomach potential is inherently capable of producing sufficient energy for the secretion of gastric juice.

SUMMARY

The potential of the "resting" body of the dog's stomach was shunted through electrodes which had a resistance of approximately the same order of magnitude as the stomach. Currents of 67.8 to 276 microamperes per square centimeter could be drawn off continuously. Evidence is presented that there is no maintained depolarization of the potential upon the withdrawal of currents of the above magnitudes.

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A COMPARATIVE STUDY OF THE OXYGEN CONSUMPTION OF THE VERTEBRATE RETINA, WITH ESPECIAL REFERENCE TO THE NUCLEO-PROTOPLASMIC RATIO

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The retina affords a number of unusual opportunities for studies on the respiratory activity of nervous tissue. Numerous contributions have been made to the literature on the gross metabolism of this tissue. Among the more recent studies are those of Adler (1935), Kodama (1936), Krebs (1929), Kubowitz (1929), Lindeman (1940), and Jongbloed and Noyons (1936).

In many respects the respiratory activity of the retina resembles the gray matter of the brain. But the retina, as a tissue, affords definite advantages over brain tissue for the metabolic studies of nerve cells. First, it probably contains a greater concentration of neural elements per unit mass than any other region in the nervous system. Secondly, it is thin enough to fall within the thickness generally required for respiratory studies of tissue in vitro, thus eliminating the necessity of slicing.

This study deals largely with the experimental analysis of the respiratory activity of nerve cells in the retina of several vertebrates. An attempt is made to secure additional information about the relative rates of nuclear and cytoplasmic respiration in neural tissue. These data provide a basis for computing the relative rates of oxygen consumption of individual nerve cells and their nuclei in the retina.

Material. The following animals, representatives of three classes of vertebrates, were used in this study:[1] Sucker (Catostomus catostomus), shiner (Notemigonus crysoleucas), chub (Leucosomus corporalis), frog (Rana pipiens), toad (Bufo americanus), necturus (Necturus maculosus), tortoise (Clemmys muhlenbergii), garter snake (Thamnophis sirtalis), and alligator (Alligator mississippiensis). All the specimens, except the tortoise and alligator, were collected locally during the spring and summer months and were used experimentally within two weeks of their capture. In each case the tissue used was the retina.

Methods. The retina was prepared for experimental purposes as follows: The animal was decapitated and the eyes removed. The eye was placed in Ringer's solution and the cornea dissected away. The lens was then removed, and with the aid of a curved forceps the retina lifted intact from its position in the eye.

The oxygen consumption was measured by the direct method of Warburg in air and at 25.3 degrees centigrade. Vessels with a capacity of approximately 5 milliliters were employed on the manometers. All measurements were made in 1 ml. of Ringer's solution, buffered with phosphates to a pH of 7.5. The Ringer's

[1] Jordan, D. S. Manual of the vertebrate animals. 13th ed.

solution was prepared each time from stock solutions of pure m/9 NaCl, m/9 KCl, and m/9 $CaCl_2$, in proportions of 100:1.5:1, respectively. Forty per cent KOH was used in the central well to absorb the carbon dioxide. The rate of oxygen consumption was computed from an average of the first 2 hours, and expressed in cubic millimeters oxygen consumed per milligram wet weight of tissue per hour.

The technique employed for counting and measuring the nuclei was similar to that used by Pearce and Gerard (1942) for frog brain tissue. In brief the procedure was as follows: After removing the retinae from the respiratory chambers they were carefully blotted and weighed. They were then transferred to small test-tubes containing exactly 2 ml. of 25 per cent acetic acid in water. Gentle shaking reduced the tissue to a fine suspension of intact nuclei and dissolved cytoplasm. One-fourth milliliter of 1 per cent methylgreen was added to stain the nuclei. Approximately 15 minutes was allowed for maximal staining. Samples were removed from the test-tube with a white cell diluting pipette and a drop of the suspension was placed under the cover glass of a hemocytometer counting chamber. The nuclei in 25 large squares were counted for each sample and at least 3 samples from each suspension were averaged. The variation between different samples of a suspension was seldom more than five per cent. The number of nuclei per milligram of retina was calculated for each suspension.

The nuclear dimensions were measured with an ocular micrometer at a magnification of approximately 450 ×. The long and short diameter of 1000 nuclei was measured for each representative of vertebrate retina studied.

The protoplasmic volume of the whole retina was measured by a method employing Archimedes Principle. The procedure consisted first, of weighing the retina in air, while suspended from a fine strand of silk thread which had been carefully oiled to reduce surface tension. The retina was then weighed suspended in a dish containing m/9 NaCl. The weight of the thread was subtracted from both weights. The volume was computed by using the formula

$$\text{Volume} = \frac{W_A - W_{NaCl}}{d_{NaCl} \times W_A} = \text{cubic millimeter/milligram of tissue}$$

W_A Weight in air.
W_{NaCl} Weight in NaCl.
d_{NaCl} Density of the NaCl solution.

It will be noted that connective and non-neural tissues are not taken into consideration when computing the values for neural elements. These corrections are difficult to determine and at best can only be estimated. When the relative number of nerve cells per unit mass of retina is compared with an equal mass of brain tissue, the retina greatly exceeds the average brain sample in cell population. This is also true for total nuclear mass per milligram of tissue. If it can be assumed that the ratio of nucleus to cytoplasm is approximately the same in the cells of the retina as in brain tissue, the greater number of neural units in the retina would considerably reduce the non-neural elements. Pearce and Gerard (1942) found that the average brain sample contained approximately 10 per cent endothelial cells, with the anterior olfactory

nucleus having as low as 6.5 per cent. In view of the greater nerve cell population in the retina it seems reasonable to assume that the amount of non-neural tissue would be somewhat lower than that in the average brain sample. Moreover, when one considers that the non-neural cells in the average frog brain sample is estimated to consume only about 0.1 per cent of the total oxygen uptake per hour (Pearce and Gerard, 1942), it logically can be considered quite unlikely that the non-neural elements would contribute any more to the total metabolism of the retina. This, coupled with the possibility of various technical errors involved in cytological measurements, would no doubt cancel out the non-neural tissue as a value to be considered in the computations.

RESULTS AND DISCUSSION. It seemed desirable, as the first step in the experimental part of this study, to determine the relative number of neural cells in the retina of the different vertebrates being studied. By counting the number of nuclei in a measured volume of tissue suspension a fairly accurate determination

TABLE 1

Nuclear count per milligram of retina ($\times 10^3$)

ANIMAL	EXPERIMENTS	MEAN NUMBER OF NUCLEI	STANDARD DEVIATION	PROB. ERROR OF MEAN
Sucker	9	592	2.8	0.61
Shiner	10	925	14	3.04
Chub	8	836	31	7.4
Necturus	8	72.9	6	1.4
Frog	10	325	5.2	1.13
Toad	9	419	14	3.1
Tortoise	8	287	22	5.3
Snake	9	708	36	8.09
Alligator	8	743	50	11.7

of the cells was obtained. The term "nerve cell," as it is frequently used, refers to all neural cells irrespective of their modification or function.

The data contained in table 1 form a summary of the nuclear count for the various vertebrate retinae studied. The mean number of nuclei represent an average of at least 25 counts on from 8 to 10 retinae.

The group as a whole displayed a rather wide range of variation in the number of nerve cells per unit mass. It should be noted that the one extreme is represented by the necturus with a count of 72,000 cells and the other extreme by the shiner with 925,000. Between these two extremes there is a fair representative distribution.

The next step in the cytological study involved the measurement of nuclear volumes. Assuming that the nucleus be either an ellipsoid or a sphere, the volume can be computed from two measured diameters. Where one diameter is greater than the other, it is assumed to be an ellipsoid and the volume can be computed from the formula, $V = \frac{\pi}{6} d^2 l$. Where the two diameters are equal, the mass is assumed to be a sphere and the volume is computed from the formula $V = \frac{\pi}{6} d^3$.

Measurements of the long and short diameters were made for 1000 nuclei from several suspensions of each vertebrate retina used in this study. Those nuclei having the same dimensions were then classified into groups and their individual as well as their total volumes computed. The total nuclear volume per milligram of retina was obtained by multiplying the mean nuclear volume per nucleus, computed on 1000 nuclei, by the mean number of nuclei per milligram of retina.

The measurements of total protoplasmic volume per milligram of retina were made upon the retina of the frog, toad, sucker, and tortoise. The values obtained in each case were all so nearly the same, within the limits of experimental error, that a mean was obtained from 14 determinations on retinae from the above four animals. The mean protoplasmic volume of the whole retina, as determined in this manner was 0.9746 ± 0.0018 cu. mm. per milligram of tissue. This value was used to compute the nucleo-protoplasmic ratio of all retinae. By dividing the total nuclear volume by the total protoplasmic volume a ratio is obtained. A summary of these data is found on table 2.

TABLE 2

A comparison of the nuclear and protoplasmic volumes

	SUCKER	CHUB	SHINER	NECTURUS	FROG	TOAD	TORTOISE	SNAKE	ALLIGATOR
	Nuc./mgm./retina. $\times 10^3$								
	592	836	925	72.9	325	419	287	708	743
Mean nuc. vol./mm.^{3} $\times 10^{-7}$	1.51	1.05	0.86	40.1	2.78	2.59	2.37	1.18	2.19
Total nuc. vol./mg. /ret.mm.^{3}	0.089	0.088	0.0795	0.292	0.106	0.108	0.068	0.083	0.175
Nucleo-protoplasmic ratio	0.093	0.093	0.082	0.300	0.109	0.111	0.070	0.088	0.180

As one would expect, there is a reasonably close relationship between the mean nuclear volume and the number of cells per milligram of tissue. When the total nuclear volume is compared with the total protoplasmic volume a few cases stand out as not conforming to the general pattern. Particular attention is directed to the necturus, alligator and the tortoise. The nucleo-protoplasmic ratio is 0.300 for the necturus, 0.180 for alligator, and 0.070 for the tortoise, as compared to an approximate ratio of 0.09 for the other retinae studied.

These findings suggest a possible approach to the question: How much does the nucleus as a mass contribute to the total oxygen consumption of the cell? If the assumption is made that the whole protoplasmic mass contributes equally to the total respiration of the cell, as claimed by Pearce and Gerard (1942), for frog brain neurones, then the ratio of nucleus to cytoplasm within a cell should not be a factor in determining its rate of metabolism. In other words, a cell with a nucleo-cytoplasmic ratio of 0.300 might have a rate of oxygen consumption of the same magnitude as one with a ratio of 0.07. On the other hand, if the nucleus and cytoplasm have appreciably different rates of metabolism the ratio of one to the other might conceivably influence the rate of the whole cell.

In order to test this assumption, oxygen consumption measurements were made on various vertebrate retinae. The data contained in table 3 summarize the results of 82 experiments on 9 different vertebrates.

It is obvious that there is little justification for comparing the mean respiratory rates of the different retinae. However, it is interesting to compare the cell populations, nuclear volumes, and nucleo-protoplasmic ratios of the various retinae with the amount of oxygen consumed by each. Such an examination reveals that there is no correlation between the number of cells and the rate of oxygen consumption. There is no more similarity in the rate of respiration between retinae having approximately the same number of cells than there is between retinae which differ greatly in cell count. This lack of correlation also exists when respiratory rates are compared to nuclear volumes and nucleo-protoplasmic ratios. It will be noted that the mean oxygen consumption of retinae with similar nucleo-protoplasmic ratios are no more comparable than those with widely different ratios. A comparison of several specific cases will illustrate this point. The necturus retina with a ratio of 0.300 and a mean rate of oxygen consumption of 0.664 cu. mm. per milligram per hour when compared with several retinae having a ratio of approximately 0.09, has a respiratory rate higher than some and lower than others. This also holds true for the alligator which has a ratio of 0.180. Moreover, the oxygen consumptions of retinae with similar nucleo-protoplasmic ratios are no more consistent.

TABLE 3

Mean oxygen consumption of vertebrate retina mm^3 per mgm. per hour

ANIMAL	EXPTS.	MEAN O_2 CONSUMED	STANDARD DEVIATION	PROB. ERROR OF MEAN
Sucker	10	0.592	0.063	0.012
Shiner	9	0.768	0.028	0.006
Chub	9	0.756	0.010	0.002
Necturus	9	0.664	0.035	0.008
Frog	10	0.467	0.027	0.005
Toad	8	0.540	0.090	0.022
Tortoise	9	0.445	0.035	0.008
Snake	10	0.569	0.035	0.006
Alligator	8	0.529	0.033	0.008

If a sufficient sampling has been made and one is justified in making the above comparisons, this evidence would seem to indicate that the whole protoplasmic mass contributes equally to the total metabolism of the retinal nerve cells. These findings are also in agreement with the findings of Pearce and Gerard (1941) for frog brain cells. If this is a reasonable assumption it should be possible to compute the oxygen consumption of the individual nuclei and in turn, the whole cell. It is a rather simple matter to compute the mean rate of respiration per cell. This can be done by simply dividing the oxygen consumed per milligram of retina by the number of nuclei per milligram of tissue. The mean oxygen consumption per cell is of little significance since it merely represents a value obtained upon a hypothetical cell. However, by employing this value in

conjunction with measurements of nuclear and protoplasmic volumes (table 2) the mean oxygen consumption of a cell of known volume can be approximated.

Before the oxygen consumption of the individual cells could be evaluated it was necessary to know their volumes. The method employed in obtaining these data postulates a nucleo-cytoplasmic ratio for each cell on the basis of values obtained for the nucleo-protoplasmic ratio per milligram of retina; hence the values for cell volumes are only relative and can only be considered as approximations. The author recognizes the limitations of this method, but because of the practical difficulty of measuring accurately the volume of a single nerve cell this procedure appeared to be a plausible solution to the problem.

TABLE 4

A comparison of the mean rates of oxygen consumption of cells and nuclei

	SUCKER	CHUB	SHINER	NECTURUS	FROG	TOAD	TORTOISE	SNAKE	ALLIG.
	Mean O_2 consumed per mgm./ret./ per hr. mm.^{3}								
	0.592	0.768	0.756	0.664	0.467	0.540	0.445	0.569	0.529
Mean O_2 consumed per cell/hr. mm.^{3} $\times 10^{-6}$*	1.0	0.90	0.80	9.11	1.44	1.29	1.55	0.80	0.71
O_2 consumed by nuc. per mgm. ret. per hr. mm.^{3}†	0.054	0.066	0.065	1.98	0.092	0.0603	0.031	0.0475	0.0296
Mean O_2 consumed per nucleus. mm.^{3} per hr. $\times 10^{-7}$‡	0.94	0.77	0.71	25.7	3.8	1.44	1.08	0.67	1.24

* $\dfrac{O_2 \text{ consumed per mgm. per hr.}}{\text{Nuclear count per mgm. retina.}}$

† $\dfrac{O_2 \text{ consumed per mgm. per hr.} \times \text{total nuclear volume per mgm. retina}}{\text{Protoplasmic volume per mgm. of retina.}}$

‡ $\dfrac{\text{Oxygen consumption of nuclear mass per mgm. retina}}{\text{Nuclear count per mgm. retina.}}$

By utilizing the data in tables 2 and 4 the oxygen consumption of nuclei of different volumes was computed. This was done according to the following formula:

$$\frac{\text{Oxygen consumption of mean nuclear volume} \times \text{volume of nucleus}}{\text{Mean volume of nuclei}}$$

The formula for computing the individual cell volumes employed the following relationships:

$$\frac{\text{Protoplasmic volume per mgm. of retina} \times \text{volume of nucleus}}{\text{Total nuclear volume per mgm. of retina}}$$

The oxygen consumption of the cells with different volumes was computed as follows:

$$\frac{\text{Oxygen consumption of the nucleus} \times \text{volume of the cell.}}{\text{Volume of the nucleus.}}$$

Table 5 is an analysis of the cellular respiration of 3 vertebrate retinae, one representative from each class. Columns 1 and 2 give the nuclear and cell volumes respectively, 3 and 4 the corresponding rate of oxygen consumption for the nucleus and cell, and 5, a percentage grouping of nuclei according to volume.

TABLE 5

The relative oxygen consumption of nuclei and cells of different volumes

NECTURUS					SHINER					ALLIGATOR				
Volume ($mm.^3$)		O_2 consumed ($mm.^3$)		No. nuc.	Volume ($mm.^3$)		O_2 consumed ($mm.^3$)		No. nuc.	Volume ($mm.^3$)		O_2 consumed ($mm.^3$)		No. nuc.
Nuc. 10^{-7}	Cell 10^{-7}	Nuc. 10^{-7}	Cell 10^{-7}		Nuc. 10^{-7}	Cell 10^{-7}	Nuc. 10^{-7}	Cell 10^{-7}		Nuc. 10^{-7}	Cell 10^{-7}	Nuc. 10^{-7}	Cell 10^{-7}	
				per cent					*per cent*					*per cent*
16.9	53.7	10.8	35.6	7	0.265	3.17	0.160	1.93	52	0.79	4.41	0.44	2.45	3
21.6	71.3	11.3	47.7	11	0.391	4.70	0.238	2.88	8	0.87	4.85	0.49	2.73	37
23.4	77.2	14.8	48.8	8	0.530	6.35	0.323	3.90	2	1.17	6.28	0.66	3.68	1
35.3	116.0	22.2	73.0	5	0.870	9.85	0.720	8.17	20	1.75	9.38	0.99	5.50	20
39.1	129.0	25.0	83.0	38	1.170	14.0	0.835	10.0	3	2.37	13.20	1.33	7.43	23
					2.370	28.5	1.48	17.8	9	2.84	15.80	1.60	8.82	4
46.6	164.0	29.7	101.0	8	3.230	38.8	1.97	23.8	4	3.23	18.00	2.00	11.10	4
53.6	177.0	34.0	112.0	4	6.960	83.5	4.25	51.1	2	4.41	24.40	2.48	13.80	2
55.7	183.5	35.4	116.0	6						6.96	38.80	3.92	21.80	5
66.2	218.5	44.3	138.0	9						8.97	49.90	5.05	28.00	1
83.0	274.0	53.0	175.0	1										
91.9	303.0	81.8	246.0											

SUMMARY

1. Cytological measurements and oxygen consumption determinations were made upon the following vertebrate retinae: Sucker, Chub, Shiner, Frog, Necturus, Toad, Snake, Tortoise, and Alligator.

2. The cell population of the different retinae was found to range from 72,900 per milligram in the necturus, to 925,000 per milligram in the shiner, with a good range of distribution between these extremes.

3. The ratio of nucleus to protoplasm was approximately 1:10 in all cases except the necturus, alligator and tortoise, which had ratios of 1:3.3, 1:5.56, 1:14.3, respectively.

4. No apparent correlation seemed to exist between the nucleo-protoplasmic ratios and the mean rate of oxygen consumption of the various retinae. This was interpreted to indicate that the whole protoplasmic mass contributed equally to the total metabolism of the cells.

5. The oxygen consumption of the individual nerve cells was computed in relation to their nuclear and cell volumes.

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THE EFFECT OF DIETHYLSTILBESTROL ON THE BLOOD PRESSURE OF NORMAL AND HYPOPHYSECTOMIZED RATS

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A lowering of the blood pressure following the removal of the hypophysis has been reported for a number of animals. Wyman and tum Suden (1) measured the blood pressure of the hypophysectomized rat by carotid cannula and found it to be subnormal. Recently Williams, Harrison and Grollman (2) have described a method by which repeated systolic blood pressures can be obtained on the unanesthetized rat and it was a purpose of this investigation to use this method and record any blood pressure changes following hypophysectomy.

Estradiol benzoate and diethylstilbestrol have been shown to elevate the blood pressure of the normal rat, in some cases, to hypertensive levels (3). In view of this action of stilbestrol in the normal rat it appeared of interest to compare its action on the blood pressure of the normal and hypophysectomized rat.

MATERIALS AND METHODS. Forty-nine male rats, weighing 250 to 340 grams and ranging from 100 to 176 days in age, were used for these experiments. Of this group, 29 were hypophysectomized. The systolic blood pressure was determined by the method of Williams, Harrison and Grollman (2). Several pressure determinations were made before hormone administration or hypophysectomy, because the first two or three determinations were usually found to be higher than normal. However, the animals readily became habituated to the apparatus and normal rat pressures exhibited little variation.

Diethylstilbestrol[2] was dissolved in sesame oil and administrated intramuscularly in daily dosages of 1 mgm. contained in 0.05 cc. of oil.

Pituitary capsules were serially sectioned, stained with Mallory's connective tissue stain and examined microscopically. Data from only completely hypophysectomized rats are presented.

RESULTS. *Normal rats.* The systolic blood pressure of normal rats fluctuated between 111 and 118 mm. Hg as an average for eleven animals over a twenty-nine day test period (fig. 1).

Injection of 1 mgm. of stilbestrol daily into nine normal rats caused a gradual rise in systolic blood pressure in eight animals. After ten injections a definite upward trend of the pressure was established and after twenty-eight days of hormone administration the average pressure of the stilbestrol treated rats was twenty millimeters above the normal. The degree of rise varied from 0 to 38 mm. in different rats. Individual cases exhibited four instances in which the

[1] Part of this investigation was carried out in the laboratory of Dr. W. W. Swingle and we are indebted to him for certain necessary facilities.

[2] The stilbestrol was generously supplied by Dr. John F. Anderson of E. R. Squibb & Sons.

pressure ranged between 145 and 153 mm., the latter being the highest pressure recorded (fig. 1).

Hypophysectomized rats. Following hypophysectomy the blood pressure dropped rapidly, being 76 mm. for an average of nine rats following a postoperative interval of eight days. At about this time the blood pressure became stabilized and maintained approximately an 80 millimeter average for the twenty-nine day investigational period. Individual systolic pressures following hypophysectomy ranged from 65 to 90 mm. and represented an average decrease in pressure of 30 to 35 mm. (fig. 2, curve *A*). We have also determined the blood pressure of nine rats following a post-operative period of one hundred eighty to

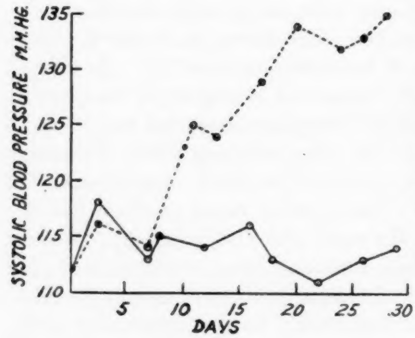

Fig. 1

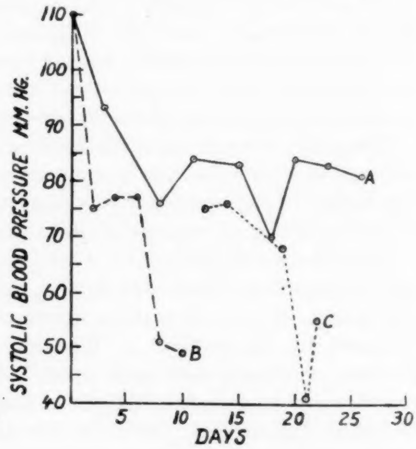

Fig. 2

Fig. 1. Effect of diethylstilbestrol on systolic blood pressure of normal rats. O——O normal untreated rats; O----O, normal rats injected with 1 mgm. of stilbestrol per day.

Fig. 2. Effect of diethylstilbestrol on the systolic blood pressure of hypophysectomized rats. Curve *A*, change in systolic blood pressure of hypophysectomized rats. Curve *B*, systolic blood pressure of hypophysectomized rats injected with 1 mgm. of stilbestrol starting the day of hypophysectomy. Curve *C*, systolic blood pressure of hypophysectomized rats injected with 1 mgm. of stilbestrol daily starting 12 days postoperatively.

two hundred twenty-two days. Several determinations were made on each rat during the course of a week and blood pressures were found to range from 71 to 83 mm.

Starting the day of hypophysectomy, seven rats were injected with 1 mgm. of stilbestrol daily for ten days. The blood pressure decline was markedly accentuated, reaching 75 mm. in three days. At the end of ten days the average blood pressure was 50 mm., which represented a pressure of 25 mm. below that of untreated hypophysectomized rats (fig. 2, curve *B*). One animal failed to survive the ten-day injection period but this appeared to approximate the maximum treatment period the rat could tolerate.

To offset the combined effect of stilbestrol and hypophysectomy, the injection of stilbestrol was withheld until twelve days after the operation at which time the blood pressure is essentially stabilized. Injection of stilbestrol in 1 mgm. daily dosages produced a marked drop in blood pressure in eight to nine days, the pressure being 20 to 30 mm. below the control level (fig. 2, curve *C*). Two of the four rats died after ten daily injections.

Discussion. The systolic blood pressure of the normal rat can be increased from 15 to 50 mm. by the daily oral administration of 1 mgm. of diethylstilbestrol, an effective rise being observed in six to ten days (3). In our experiments the intramuscular injection of the same dosage into normal rats caused an increase in blood pressure but the response was less rapid, being first observed after ten days and definitely established after twenty days. The average pressure increase was 20 mm. and the maximum response was a 38 mm. rise in pressure. The specific manner in which this effect is accomplished is unknown but some possibilities suggest themselves, such as a direct influence on the vascular system, since Reynolds and Foster (4, 5) have observed an effect of estrogen on peripheral circulation although without a constant effect on blood pressure. The adrenal glands may influence the pressure response since these organs were markedly hypertrophied, or the kidney may play an influential rôle in that sterols have been shown by Selye (6) to alter the kidney. Grollman et al. (3), suggest the hypertensive effect is due to renal injury since the elevated blood pressure can be reduced with renal extracts.

In hypophysectomized rats the average systolic blood pressure decreases 30 to 35 mm. during the first ten days post-operatively and maintains approximately the same level for as long as 222 days. However, administration of stilbestrol to hypophysectomized rats causes an initial pressure decrease of 30 to 35 mm. in three days with a further decline of about the same proportion occurring after eight to ten days. That the action of stilbestrol is not merely accentuating the effect of hypophysectomy is shown by a similar reduction in blood pressure in animals in which injections were begun twelve days after the operation. The lowered pressure need not be correlated with death of hypophysectomized rats as we have recorded pressures of 80 to 90 mm. in animals that died during the following twenty-four hours. Thus, under the conditions of these experiments there is no evidence for a direct effect on the vascular system to raise the blood pressure although the toxic effect may overshadow any pressor action. The toxic effect of stilbestrol, in the dosage used, on hypophysectomized rats is further indicated by a maximum ten to fifteen days' survival period whereas in the normal rat a pressor action is evident in this time. The toxic effect of stilbestrol has been observed in adrenalectomized rats (7).

SUMMARY

1. The daily injection of stilbestrol into normal rats produces a gradual rise in systolic blood pressure, reaching hypertensive levels in many cases.

2. The systolic blood pressure of the untreated hypophysectomized rat decreased, on the average, 30 to 35 mm. below normal within ten days after hypo-

physectomy. Rats hypophysectomized for seven months had a blood pressure stabilized at this low level.

3. The injection of hypophysectomized rats with stilbestrol produced a fall in blood pressure below that of operated controls.

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THE EFFECT OF *l*-ASCORBIC ACID ON EPITHELIAL SHEETS IN TISSUE CULTURE[1]

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The rôle of *l*-ascorbic acid as an activator for the growth of tissues is well recognized. Baker (1) found that the addition of 0.1 to 0.5 mgm. per cent ascorbic acid to a culture medium containing serum, peptone, etc., resulted, after about 2 weeks, in stimulating the proliferation of fowl monocytes. She ascribed the latent period of two weeks to the vitamin reserves in the original tissue. Vogelaar and Ehrlichman (2), by using a largely synthetic medium, found that the stimulating action of ascorbic acid on the growth of mouse sarcoma tissue was appreciable within several days.

Another angle of approach is that of Wolbach and his co-workers (3, 4, 5) who assigned the activity of vitamin C to the elaboration and maintenance of collagenous material which they regarded as cell-binding substances. The deficiency of this interstitial substance in scorbutic animals and recovery on feeding with vitamin C offered a strong argument for this view. Further substantiation has been presented by Jeney and Törö (6) who reported the beneficial effect of ascorbic acid on the formation of fibers in tissue cultures of fibroblasts. Wolbach and Bessey (7), in a recent review, refer to several papers on the beneficial effect of ascorbic acid in the healing of wounds in experimental human ascorbic acid deficiency.

No distinction seems to have been made between collagenous material as a supporting tissue and the actual intercellular cement which binds epithelial cells together. The maintenance of the latter has been shown (8, 9) to be directly proportional to the concentration of calcium ions in the medium. It was of interest to determine whether this effect is stimulated by the presence of *l*-ascorbic acid or whether the latter acts more directly on intracellular reactions.

The feature of the experiments described in this paper is the use of tissue cultures with well-formed epithelial sheets washed free of the diffusible components of the normal plasma medium and then mounted in various experimental media, some of which contained and others lacked *l*-ascorbic acid.

The tissues used were kidney and intestine from 10 day chick embryos, also kidney and parotid gland from 20 day embryos of the guinea pig which is known to depend on external sources for its supply of vitamin C.

The culture medium consisted of 1 part fowl plasma, 3 parts serum (normal guinea-pig serum for guinea-pig tissues and chick serum for chick tissues) and 1 part guinea-pig embryo or chick embryo extract. This clots within 2 or 3 minutes after mixing and forms a soft coagulum around the tissue fragment.

The selected tissues, cut into small fragments (ca 1–2 mm.), were placed in a

[1] Aided by funds from the International Cancer Research Foundation, Philadelphia.

drop of the freshly prepared medium on a coverslip attached to a larger coverslip by a drop of moisture. The larger coverslip, carrying the smaller slip, was then inverted and sealed over a depression slide and the preparation incubated at the appropriate body temperature. This method is known as the double coverslip method (10, 11) and permits transferring the smaller coverslip aseptically and washing the culture undisturbed in its clotted medium.

The experimentation was started after about 48 hours of incubation when the epithelial sheets were extensive. The first step, which involved all the cultures, was to wash them in buffered Tyrode solution[2] for 30 to 45 minutes during which the bathing solution was changed every 4 to 5 minutes. This removed the diffusible ingredients normally present in the clotted medium, but did not affect the fibrin clot which supported the explants and the cellular outgrowths surrounding them. The washing did not disturb the cellular tissues but caused a cessation of growth.

In order to test the possible rôle of *l*-ascorbic acid on intercellular cement formation, the washing solution, in some cases, was Tyrode solution lacking calcium salts. This removed Ca ions from the culture until the cells of the sheets became more or less separated by a dissolution of the intercellular cement (9).

TABLE 1

	IMMERSION FLUID	OBSERVED EFFECTS
I	Tyrode solution alone	18 hours: Initial stages of deterioration; granulation of cells and lack of growth 48 hours: No improvement
II	Tyrode + cortical extract (1:10,000)	
III	Tyrode + cortical extract (1:1000)	
IV	Tyrode + ascorbic acid alone (10 mgm. in 100 cc.)	18 hours: Good condition
V	Tyrode + cortical extract (1:10,000 or 1:1000) and ascorbic acid (10 mgm. in 100 cc.)	48 hours: Good condition

The second step was the addition to the culture of the various experimental media. Two general procedures were used to test the effect of *l*-ascorbic acid on the washed cultures. One was to compare the effect of plasma or serum from a normal guinea pig with that of plasma or serum from a scorbutic guinea pig. The other was to compare the effect of *l*-ascorbic acid[3] in Tyrode with that of Tyrode alone. In all experiments of this series, the solutions in the *l*-ascorbic acid were used immediately after preparation. The scorbutic guinea pigs were furnished through the courtesy of Dr. R. L. Zwemer, the College of Physicians and Surgeons, Columbia University[4].

[2] The formula used for the Tyrode solution was: NaCl 8.00 grams, KCl 0.20 gram, $CaCl_2$ (anhydrous) 0.20 gram, $MgCl_2$ 0.10 gram, glucose 1.0 gram, NaH_2PO_4 0.05 gram in distilled water to 1000.00 cc. The solution was adjusted to pH 7.6 by the addition of $NaHCO_3$.

[3] The *l*-ascorbic acid was a water soluble crystalline product listed as Ascorbic acid vitamin C, U.S.P. Merck.

[4] The guinea pigs were fed on a diet of oats and showed first signs of scurvy, one on the fifth and the other on the seventh day. Blood for the experiments was obtained on the 27th day when the guinea pigs were significantly ill.

Guinea-pig tissues were used in the experiments with guinea pig plasma and serum, chick embryo tissues in the experiments with the Tyrode solutions.

1. *Experiments with scorbutic plasma and serum.* Forty-eight hour cultures of guinea-pig epithelium were bathed in frequent changes of Ca-free Tyrode solution until the cells of the epithelial sheets were loosened from one another and had become rounded. This occurred in from 20 to 30 minutes. A drop of plasma from a scorbutic guinea pig was then added to the washed cultures. The control cultures were treated in the same way, using plasma from a normal animal.

After 3 hours the sheets had reformed equally well in the experimental and the control cultures. The rounded cells spread out and, when contiguous, cohered into homogeneous sheets with the usual absence of visible cell outlines. Moreover, the sheets remained firmly attached to the culture substrate.

The difference between the effect of the two types of plasma became apparent after 15 hours of incubation. In the cultures with scorbutic plasma, the sheets, although coherent, showed no increase in area and the cells gave signs of deterioration, becoming granular especially along the margins of the sheets where the cells tended to round up and become clumped. On the other hand, the sheets of the cultures with normal plasma grew considerably and appeared normal and healthy.

Similar results were obtained by immersing the cultures in normal and in scorbutic guinea pig sera where they were left for 3 hours at body temperature and then remounted over depression slides each in a hanging drop of the serum in which they had been immersed.

2. *Experiments with l-ascorbic acid in physiological salt solution.* Forty-eight hour cultures of chick epithelium, previously washed in Tyrode, were immersed, some in Tyrode solution alone for controls, and others in Tyrode solution containing 10 mgm. in 100 cc. *l*-ascorbic acid freshly prepared.[5] One series of the cultures had been washed in the normal calcium-containing Tyrode and another series, in calcium-free Tyrode until the cells had begun to separate.

The sheets with partially separated cells recovered rapidly irrespective of the presence or absence of the *l*-ascorbic acid in the final calcium-containing medium. However, about 12 hours later the sheets of the cultures in the salt solution lacking *l*-ascorbic acid were unhealthy and showed signs of degeneration within 18 to 24 hours while those in solutions containing it were healthy and showed active growth for the 96 hours' duration of the experiment. The cultures which had not been previously exposed to the Ca-free solutions gave similar results.

3. *Experiments with adrenal cortical extract.*[6] Experiments were also made to test the action of the whole extract of the adrenal cortex in the presence and absence of *l*-ascorbic acid on the epithelial sheets of the chick embryo kidney and

[5] No attempts were made to test the effect of varying the concentration of the *l*-ascorbic acid, although in several experiments a concentration of 5 mgm. in 100 cc. was used with no pronounced differences being noted. The higher concentration was routinely used because of the ease with which the acid is destroyed on oxidation.

[6] The extract was furnished through the courtesy of Dr. E. C. Kendall. One cubic centimeter of this extract represents extractable components of 75 grams of adrenal cortex.

intestine. The cultures were divided into five lots and immersed in media as indicated in the table. They were then removed from the media after four hours and sealed over depression slides in hanging drops of their respective experimental fluids. The results are shown in the accompanying table, viz., that *l*-ascorbic acid stimulates epithelial growth in Tyrode solution irrespective of the presence or absence of the cortical extract.

Discussion. The experiments described in this paper fully confirm the results obtained by Baker and by Vogelaar and Ehrlichman by demonstrating the importance of *l*-ascorbic acid for stimulating the growth of epithelial tissues. Our more rapid detection of the effect is presumably due to the method of removing effective traces of the *l*-ascorbic acid before starting the experimental procedure.

The failure of *l*-ascorbic acid to affect the development and function of the intercellular cement of parotid gland, intestinal and renal epithelium does not necessarily counter the findings of Wolbach and his co-workers. Wolbach had shown that vitamin C is essential for the formation and maintenance of interstitial matrices, more particularly of dentin, bone and of collagen in connective tissues. Menkin, Wolbach and Menkin identified the action described by Wolbach with that of crystalline *l*-ascorbic acid. The non-essentialness of *l*-ascorbic acid for the production and effectiveness of the material which causes cells to adhere together indicates that the intercellular cement proper is of a different order from materials of collagenous nature. Cells which depend upon a cement for making them stick together can generate such a cement in the absence of *l*-ascorbic acid. That which is required to render the cement effective is the presence of ionic calcium which presumably fixes the cement by converting it into a non-dispersible complex. However, cellular reactions of multiplication, growth and movement are definitely accelerated by the presence of *l*-ascorbic acid.

The experiments with the adrenal cortical extracts were made because of the possibility that these extracts may exert an additive effect to that of the ascorbic acid. Experiments in this laboratory (unpublished) indicate that the extract of adrenal cortex stimulates the secretory activity of kidney tubules in tissue culture.

SUMMARY

1. Tissue cultures were made of kidney and parotid gland from guinea pig embryos and of kidney and intestine from chick embryos. The action of *l*-ascorbic acid was tested on the growing epithelial sheets. Cultures treated with plasma and serum from scorbutic guinea pigs ceased to grow but the epithelial sheets did not lose their coherence. Ultimately the cells deteriorated.

2. The presence of *l*-ascorbic acid was found to be non-essential for re-establishing the cohesion of epithelial sheets previously separated by the lack of calcium in the medium. The intercellular cement upon which this cohesion depends must, therefore, be of a different order from the interstitial matrices which,

Wolbach and others have found, depend upon the presence of ascorbic acid for their elaboration.

3. Tissue cultures of epithelium, washed free of the water-diffusible ingredients of the plasma medium and transferred to a buffered salt and glucose solution, remained healthy and active only when *l*-ascorbic acid was present in the solution.

4. The addition of cortical extract either in the presence or absence of *l*-ascorbic acid exerted no observable effect on the viability of tissue cultures.

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EFFECT OF HEPARIN ON THE VASOCONSTRICTOR ACTION OF SHED BLOOD TESTED BY PERFUSION OF THE RABBIT'S EAR

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Convincing indirect evidence in favor of the humoral hypothesis of hypertension has made it important to measure quantitatively the presence of specific vasoconstrictor substances in the circulating blood of hypertensive animals and man. For this purpose Page (1, 2) perfused the surviving rabbit's ear with a mixture consisting of equal volumes of Ringer-Locke solution and defibrinated blood from a nephrectomized dog. He found that injecting serum (2) or heparinized plasma (1, 2) from a normal dog or human being into the perfused ear had little or no effect on the blood vessels of the ear, while serum or heparinized plasma from a hypertensive dog or human being was uniformly constrictor. Our attempts to use this technique proved difficult chiefly because of immediate, and irregularly increasing, vasoconstriction which appeared as soon as the diluted, defibrinated blood entered the ear. Moreover, when the constrictor activity of blood plasma or serum from hypertensive and normal animals and man was assayed, the results were highly inconsistent.

Many investigators have reported that serum and defibrinated blood constrict intensely the vessels of isolated perfused tissues (reviewed by Janeway et al. (3) and Amberson (4)). It has been suggested (3) that a constrictor substance is liberated by disintegration of platelets in the course of coagulation. It seemed possible that defibrinated blood as a perfusing fluid, and serum or plasma as samples for assay, might have produced inconsistent results with the rabbit's ear because of varying amounts of powerful vasoconstrictor substances which they contained by virtue of coagulation.

Therefore the present study was arranged *a*, to determine whether defibrinated blood per se produced vasoconstriction in the rabbit's ear; *b*, to observe whether preventing coagulation by large doses of heparin would avoid this constrictor action and thereby produce a more physiological preparation, and *c*, to compare under these conditions the vasoconstrictor properties of defibrinated blood, serum and heparinized plasma from rabbit, dog and man as a necessary step toward using the perfusion method to measure the constrictor activity of blood removed from normal and hypertensive animals and man.

Methods. a. *Initial technique.* In the first observations, the original technique described by Page (1, 2, 5) was followed. The rabbit's ear was severed rapidly and cleanly with a razor blade and the central artery was immediately cannulated by means of a gauge 21 blunted needle, tied in place by a fine ligature. Within two or three minutes after being severed, the ear was in place in the incubator at 39°C. and the perfusion was started.

The fluids used for perfusion were *a*, Ringer-Locke solution buffered by phosphate to pH between 7.3 and 7.4, and *b*, a mixture consisting of one part of defibrinated blood and either one or two parts of Ringer-Locke solution. Defibrinated blood was prepared by stirring whole blood slowly for 30 minutes with several small glass rods rounded at the ends to avoid injuring the corpuscles. After dilution with Ringer-Locke solution the defibrinated blood mixture was filtered through cotton gauze. Both fluids were prepared immediately before use, warmed to 39°C. and placed in suitable flasks in the incubator before the ear was severed.

Intermittent pressure was applied to the surface of both fluids in separate reservoirs by compressed air from a valve rotating about 60 times per minute in conjunction with a reservoir and leak. This pulsatile perfusion pressure could be adjusted between 20/10 and 140/110 mm. Hg. Connections were arranged so that transfer from Ringer-Locke solution to defibrinated blood could be made without any interruption of flow.

Immediately after the ear was mounted the buffered Ringer-Locke solution was first perfused at a pressure of 40/30 mm. Hg or less until rapid and constant flow indicated that the initial vasoconstriction due to trauma had disappeared and that the ear was properly fixed in its final position. Then perfusion by defibrinated blood was started. The perfusion fluid entered the central artery, passed through the vessels of the ear and flowed from the cut veins at the base of the ear, to be collected in a funnel from which it passed dropwise over the terminals of an electrical drop recorder. A kymograph record showed time in minutes, mean perfusion pressure and the number of drops. Fifteen drops had a volume of 1.0 cc. The air within the incubator was kept moist by means of filter paper or cotton saturated with water.

b. *Modified technique.* Owing to increasing and variable vasoconstriction observed with this initial method, heparinized blood was then used in place of defibrinated blood. Between 35 and 45 cc. of blood was withdrawn from the heart of a rabbit into a 50 cc. syringe containing 1.2 cc. Connaught heparin (1000 units per cc.). Blood and heparin were mixed throughly by inverting the syringe several times. The heparinized blood was diluted immediately with two volumes of Ringer-Locke solution and filtered through cotton gauze. This perfusion mixture was also often constrictor, as will be described under "observations", and the following modifications were finally adopted routinely. It appears that each factor is essential because omission of any one was followed sooner or later by grossly irregular results.

1. *Thorough heparinization.* An excess of heparin was used at each step in preparing the perfusing fluid to avoid coagulation as completely as possible. One and five-tenths cubic centimeter of Connaught heparin solution was injected into the donor rabbit intravenously, fifteen minutes before blood was drawn. For rabbits weighing more than 3 kilos, 1.8 cc. were used. Fifteen minutes later, between 40 and 45 cc. of blood were withdrawn by cardiac puncture into a syringe containing 1.2 cc. of heparin solution and mixed as usual. This blood was then diluted immediately with two volumes of warm Ringer-

Locke solution to which heparin solution had previously been added in the amount of 0.3 cc. in 100 cc. The mixture was filtered through cotton gauze previously saturated with heparinized Ringer-Locke solution and then placed in the incubator ready for perfusion. As an additional safeguard during perfusion, 0.3 cc. heparin solution was added at the end of 30 minutes tc such blood as still remained in the reservoir awaiting perfusion. Blood prepared in this way will be called "pre-heparinized blood."

2. *Oxygen and carbon dioxide.* A mixture of 95 per cent oxygen and 5 per cent carbon dioxide was led to the perfusion flask and was bubbled very slowly through the perfusing mixture to keep the erythrocytes from settling in the reservoir and to approach normal gaseous equilibrium.

3. *Precautions against cooling.* For dependable results it proved necessary to make all injections without opening the incubator. This was accomplished by piercing the rubber tube, which led the perfusion fluid to the ear, with a minute T-cannula consisting of a gauge 20 needle with gauge 20 side-arms. Rubber tubing with 0.5 mm. bore was passed from these two side-arms to the outside of the incubator through the wall. To one of these tubes was connected the syringe for making injections while the other was closed with a clamp. Prior to each injection the clamp was opened and the solution to be tested was washed from the syringe through one rubber tube past the base of the T-cannula, and out through the other rubber tube. The solution having thus been brought to the base of the T-cannula, the clamp was closed and exact volumes could be injected into the perfusion fluid from outside the incubator without chilling the ear.

4. *Careful cleansing.* According to Janeway (3) the powerful constrictor substance produced in the course of coagulation of blood is destroyed by alkali and dissolved by alcohol. Therefore, after each experiment, all glassware and tubing were completely dismantled and not only thoroughly washed with soap and water but soaked at least ten minutes, and preferably an hour, in 95 per cent alcohol to remove any vasoconstrictor material produced by traces of blood left in the tubing or its joints. All syringes and needles were cleansed similarly. At intervals, also, all parts were boiled in saturated sodium bicarbonate solution. After each cleansing, fresh Ringer-Locke solution was washed through the entire apparatus in large quantities.

OBSERVATIONS. 1. *Comparative effects of defibrinated, heparinized, and "pre-heparinized" blood on vascular tone in the rabbit's ear.* The differences in the vasoconstrictor activity of three types of shed blood are illustrated in figure 1. In these observations the ear was perfused as usual with Ringer-Locke solution for 5 or more minutes until the rate of dropping was rapid and constant (not shown in fig. 1). At zero time the perfusion with Ringer-Locke solution was stopped and the blood mixture began entering the ear. The rate of dropping was observed for 60 minutes or until perfusion was practically stopped by tight vasoconstriction. Perfusing pressure remained constant throughout so that decrease in drop rate indicated constriction.

When defibrinated blood entered the ear at a pressure of 40/30 mm. Hg, the

whole ear became pink but almost immediately turned pearly white as vasoconstriction reduced the rate of flow almost to zero. The cessation of flow was sudden enough to suggest mechanical obstruction by thrombi. That this was not the case was demonstrated by perfusing alternately with defibrinated blood and with Ringer-Locke solution. For three ears, Ringer-Locke solution at a pressure of 40/30 mm. Hg flowed initially at rates between 38 and 45 drops per minute. Changing to defibrinated rabbit's blood reduced the perfusion rate within two minutes to between 1 and 3 drops per minute. Returning to Ringer-Locke solution re-established perfusion at rates between 35 and 45 drops per minute but complete recovery required 10 minutes. A second and third shift to defibrinated rabbit's blood again reduced the drop rate to between 0 and 2

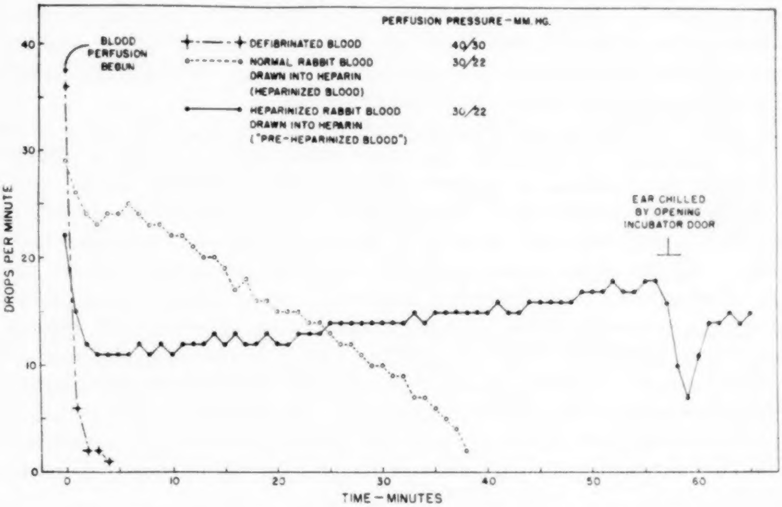

Fig. 1. Effect of perfusion by defibrinated blood, heparinized blood and "pre-heparinized blood" on the tone of the vessels of the rabbit's ear. To right is shown effect of cooling the ear.

drops per minute after which return to Ringer-Locke solution after about 10 minutes restored the original perfusion rate. This repeated reversibility indicates that slowing of flow was due to constriction rather than to mechanical obstruction by thrombi.

As shown in figure 1, when perfusion was performed with heparinized blood, the rate of flow decreased somewhat at the very beginning as would be expected because of the greater viscosity of the blood mixture as compared to Ringer-Locke solution. Thereafter for ten to twenty minutes a fairly adequate perfusion rate was observed and the ear looked generally pink and healthy. Shortly, however, the perfusion rate began to fall more or less regularly. The central artery and veins gradually became smaller until finally the ear was

pearly white, perfusion rate was low and the end result resembled that produced much more rapidly at these low pressures by defibrinated blood.

In contrast to these mixtures "pre-heparinized blood" (solid line, fig. 1) produced the usual preliminary drop in perfusion rate due to change in viscosity of the perfusing fluid, but the ear remained pink, and the central artery and marginal veins stayed moderately dilated. Moreover, the perfusion rate remained relatively high and constant at 12 to 20 drops per minute with perfusing pressures not above 40/30 mm. Hg.

Rabbit's ears perfused at low pressure with "pre-heparinized blood" proved to be extremely sensitive to minute changes in temperature. If the incubator door was opened briefly, a fall of 3 or 4°C. in air temperature often produced a 50 to 80 per cent drop in perfusion rate lasting from 4 to 6 minutes, as shown to the right in figure 1. When control injections of Ringer-Locke solution were made by opening the incubator door there usually followed a constriction which was due not to the fluid injected, but to coincident cooling. Avoiding this artefact is highly important in assays of constrictor action.

In early attempts to apply the method of Page (1, 5) to the assay of constrictor substances in human or dog blood many types of perfusion mixtures, containing serum, plasma, or washed cells, were tested. Results similar to those for defibrinated rabbit's blood were obtained with rabbit serum, defibrinated dog's blood, and with some samples of heparinized blood from either dogs or rabbits. Results more or less similar to those for heparinized rabbit's blood were observed with most samples of heparinized dog's blood, washed rabbit or dog erythrocytes resuspended in Ringer-Locke solution or with quickly separated plasma from heparinized dog's or rabbit's blood. To obtain a drop rate of 15 per min. pressures of 50/30 up to 125/100 were often necessary, indicating greatly increased tone due to the perfusing fluid itself. Defibrinated blood, heparinized blood and heparinized plasma obtained from rabbits or dogs nephrectomized 24 or 48 hours previously yielded results similar to those obtained with blood from normal animals.

These observations all indicated that, as in other isolated tissues (4), the perfusion of the isolated rabbit's ear with defibrinated or simple heparinized blood is accompanied by vasoconstriction. To avoid this effect it is essential to guard against coagulation at every step. To determine to what extent this error might interfere with the assay of pressor substances of renal or other origin in the circulating blood, various types of blood samples from rabbit, dog and man were tested for their constrictor activity by injecting small volumes such as are used for assay.

2. *Vasoconstrictor effects of small amounts of shed blood or its fractions.* In these observations the ear was perfused at low pressure with "pre-heparinized" blood. After a suitable control period, usually 5 to 10 minutes, 0.2 cc. of shed blood or its fractions was injected through the T-cannula into the perfusion mixture as it passed through the rubber tubing immediately before entering the central artery of the ear. Rabbit's blood was used in this series of experiments to eliminate effects due to species differences. Perfusion pressure was kept constant throughout each experiment.

Figure 2 illustrates typical results of numerous observations of this kind. The constriction produced by simple cooling of the ear is shown in figure 2A. Control injections of Ringer-Locke solution (fig. 2B) had no effect on perfusion rate providing the injections were made from outside the incubator. Full strength Connaught heparin solution, as supplied in ampules commercially, did not affect vascular tone when it was injected alone in volumes of 0.2 (fig. 2C) or even 0.6 cc. Defibrinated rabbit's blood produced immediate and complete constric-

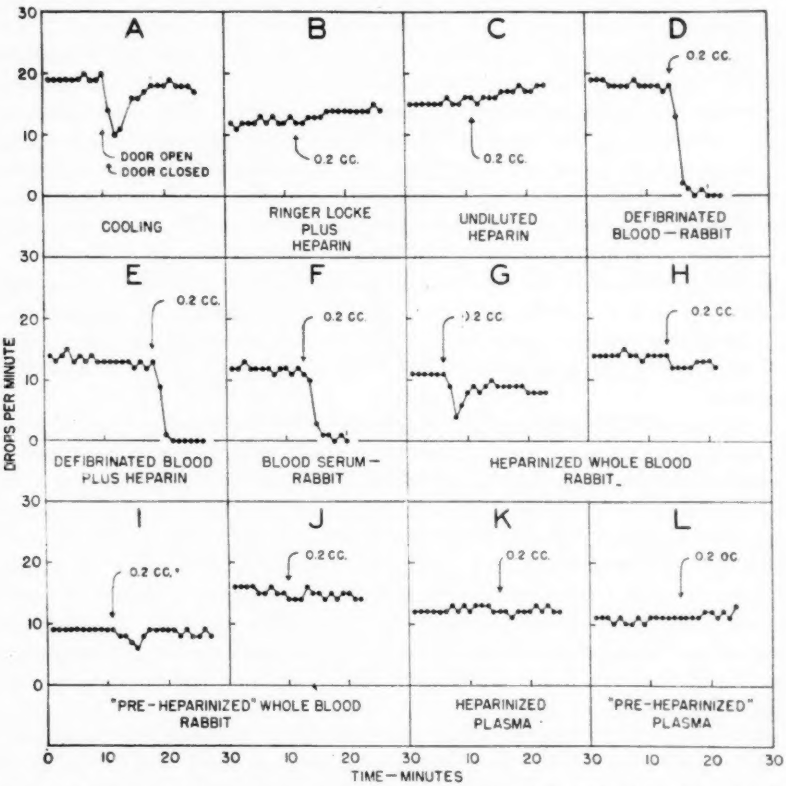

Fig. 2. Effect of cooling, heparin, and of certain types of shed blood on the tone of the vessels of the rabbit's ear.

tion (fig. 2D) which lasted at least 7 or more minutes in 6 observations. Heparin, 0.5 cc., was then added to 10 cc. of defibrinated blood and the mixture was incubated for 30 to 45 minutes without affecting the intense constriction produced by injecting 0.2 cc. (fig. 2E). This indicates first, that once vasoconstrictor substance had been released by coagulation, heparin did not neutralize or destroy it, and second, that heparin per se had no detectable dilator effect under these conditions. Blood serum (fig. 2F) also produced complete constriction and cessation of flow. Both defibrinated blood and serum changed the

color of the ear from the normal pink to waxy white as the previously dilated arteries and veins narrowed and then became imperceptible.

Heparinized whole blood, i.e., blood drawn from the heart of a normal rabbit into a syringe containing heparin, was slightly constrictor (fig. 2, G and H) in almost every instance. "Pre-heparinization" of the donor rabbit diminished still further the development of constrictor activity in whole blood in that doses of 0.2 cc. had very slight or no effect on perfusion rate (fig. 2, I and J).

Constrictor activity was least in plasma separated immediately after the blood was withdrawn from the heart of a normal or a heparinized rabbit (fig. 2, K and L). In preparing this "heparinized plasma" (fig. 2K) and "pre-heparinized plasma" (fig. 2L), blood from the heart of a normal or heparinized rabbit respectively was drawn into a syringe containing the usual 1.2 cc. of heparin solution. After mixing, the blood was rotated in plastic (Lusteroid) tubes in an angle centrifuge at high speed for 5 minutes. The upper layer of plasma was decanted into another tube and centrifuged again for 10 minutes. This was decanted and placed in the incubator for injection. As shown in figure 2K and L, constrictor substances were usually absent. If Janeway's suggestion that disruption of platelets is responsible for the vasoconstrictor activity is correct, it would be expected that this quickly separated, and doubly centrifuged, plasma would have least effect on the tone of the perfused vessels because most of the platelets are separated from the plasma before there is opportunity for gradual disintegration.

3. *Quantitative comparison of the constrictor activity of samples of rabbit's blood collected in various ways.* Full curves for rabbit's serum, heparinized blood and plasma from heparinized blood are shown in figure 3. After injection, flow quickly reached a minimum followed by gradual recovery. Perfusion pressure remaining constant, the total constrictor effect is indicated by the degree to which rate of flow is diminished and by the time required for flow to return to the control level. The scale for charting was always one inch for 10 minutes and one inch for ten drops. Rates of flow were kept as closely as possible between 10 and 15 drops per minute by adjusting pressure between 26/16 and 40/30 mm. Hg at the very beginning of each experiment and the pressure was kept constant thereafter for each ear.

Under these conditions the whole effect of a given injection can be expressed most simply by measuring the area enclosed between the observed rate of flow (solid lines, fig. 3) and the expected rate of flow without injection (dotted lines, fig. 3). Errors in this schematic method of expressing constrictor activity were relatively unimportant compared to the magnitude of the observed differences which were quite obvious to simple inspection of the original charts. Thus in figure 3 the greatest constrictor effects produced by plasma from heparinized blood and by heparinized whole blood were clearly much less than the smallest constrictor effect produced by serum in like dosage. The areas given in table 1 are used to avoid publishing many figures of the curves themselves.

Table 1 shows the average effects of control injections (Ringer-Locke solution) and of variously prepared samples of blood. The average intensity and duration

of constriction is indicated to the left while the last column to the right gives the average area of the curves showing change in flow of perfusate. The negative sign shows that the sample produced constriction and reduced flow; the figures in parentheses indicate the range of variation.

Control injections of Ringer-Locke solution had no effect. Defibrinated blood and serum were uniformly highly constrictor. It seemed possible that if blood were removed from the donor rabbit without using any anticoagulant and

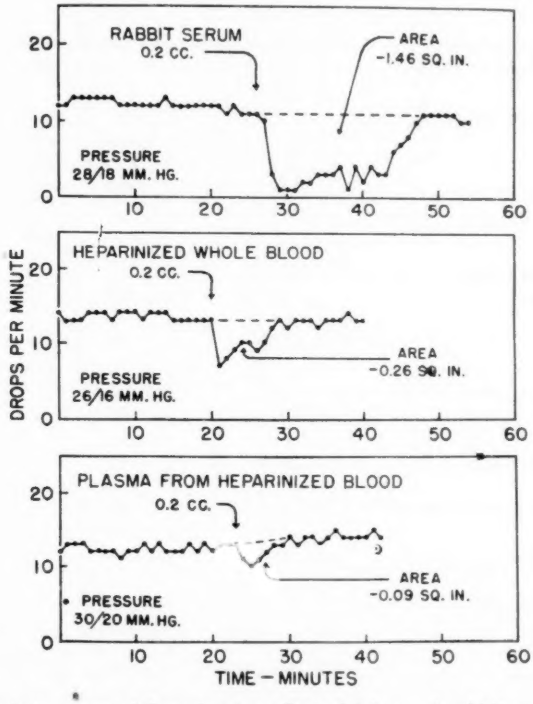

Fig. 3. Complete curves of flow showing effect of 0.2 cc. of rabbit serum, heparinized whole blood and plasma from heparinized blood on flow through the vessels of the rabbit's ear. Also shown is method of computing constrictor effect by area. For serum, 1.46 sq. in. was the *smallest* area observed while for heparinized blood and plasma, respectively, 0.26 and 0.09 sq. in. were the *greatest* areas observed.

injected within one minute or less, constrictor action might be avoided because in this brief period the blood remained fluid and did not have time to coagulate. As shown in table 1 the intensity and duration of constriction were greatly reduced but the areas of the curves were too variable to permit accepting this method as a dependable one for assay purposes. Samples of heparinized and pre-heparinized whole blood, even after standing in the incubator for one hour, were approximately one-tenth as constrictor as serum and roughly one-half as

constrictor as whole blood injected immediately. The variations were still marked.

On the contrary, samples of plasma separated rapidly by centrifugation from heparinized or "pre-heparinized" blood were usually inert and only occasionally very slightly constrictor. The greatest areas observed with these samples of plasma were 0.09 and 0.02 sq. in. respectively and represent changes of doubtful significance because control curves may show variations of this grade. The

TABLE 1

Effects of 0.2 cc. injections of rabbit's whole blood, serum and plasma on vascular tone in the rabbit's ear perfused with "pre-heparinized" rabbit's blood

1	2	3	4	5	6
	NUMBER OF INJECTIONS	AVERAGE RATE OF FLOW, CONTROL PERIOD	MAXIMAL REDUCTION OF FLOW AFTER INJECTION	AVERAGE TIME FOR FLOW TO RETURN TO NORMAL	AREA OF DECREASED FLOW CURVE
		drops per min.	*per cent*	*mins.*	*sq. in.*
Control injections Ringer-Locke soln	5	17	0	0	0.00
Defibrinated blood	6	17	100	9*	−1.61* (1.05–2.66)
Serum	5	11	98	28	−1.95 (1.46–3.85)
Whole blood injected within one minute	17	13	52	6	−0.25 (0.00–0.81)
Heparinized whole blood	8	11	43	5	−0.14 (0.00–0.26)
"Pre-heparinized" whole blood	7	12	42	5	−0.15 (0.00–0.39)
Plasma from heparinized blood	4	14	9	2	−0.04 (0.00–0.09)
Plasma from "preheparinized" blood	6	14	1	0.3	−0.01 (0.00–0.02)

* Minimum figure since recovery had not even started when the observations were terminated at 7 to 11 minutes. For serum and all other blood samples areas are based on the entire curve, continued until the control rate of flow returned.

average areas for heparinized and "pre-heparinized" plasmas were even smaller, 0.04 and 0.02 sq. in. respectively.

It seems clear that "pre-heparinized", quickly separated plasma has less constrictor activity than any other type of blood sample from the rabbit. Preheparinization is expensive and, for man, impracticable. Quickly separated, heparinized plasma is almost as inert and therefore observations on these samples were extended to the dog and man.

4. *Comparison of constrictor activity of defibrinated blood, serum and quickly separated heparinized plasma from rabbit, dog and man.* To ascertain the applicability of the rabbit's ear technique for the study of bloods of other species

the comparison in table 2 was made. Blood was removed from superficial veins of dogs or man and then treated as described above. It is obvious that defibrinated blood and serum of all three species are highly constrictor and that heparinized plasma is almost inert. In the experiments with dog plasma, 4 injections of heparinized plasma from a single animal were slightly constrictor on one day, but the next day, technique being presumably identical, a second sample of heparinized plasma was entirely inert in 4 injections. The reason for this difference is not clear, though probably due to early and slight coagulation. Even when this observation is included the average constrictor action of dog's heparinized plasma is only one-twentieth that of serum.

TABLE 2

Comparison of effects of defibrinated blood, serum and heparinized plasma from rabbit, dog and man

	RABBIT	DOG	MAN
Defibrinated blood			
No. obs.	6	9	9
Constr. eff. in sq. in.	−1.61*	−3.48	−1.60
Serum			
No. obs.	5	7	11
Constr. eff. in sq. in.	−1.95	−5.05	−3.83
Heparinized plasma			
No. obs.	4	19	16
Constr. eff. in sq. in.	−0.04	−0.24† (−0.01)	−0.07

* See footnote, table 1.

† Including one divergent experiment. Figures in parentheses show result when this one experiment is excluded.

Discussion. To perfuse isolated tissues or organs under optimal physiological conditions requires the use of oxygenated blood under pulsating pressure. Yet practically all investigators have found that shed blood after a short time induces an intense vasoconstriction which has been lessened by inserting the lung (6, 7, 8, 9, 10), liver or spleen (11) into the perfusion circuit, or by adding ergotoxine to the perfusate (7, 11). Such methods are not suitable for assay of pressor substances because of complicated technique and partial effectiveness in the first instance and because of transient pressor action of ergotoxine itself in the second instance. The hypothesis that defibrinated blood flows with great difficulty owing to mechanical block by coagulated particles (12) has been disposed of by Bayliss and Ogden (7) who found that filtration through cambric did not ensure rapid flow and by Janeway (3) who described a diffusible, heat stable constrictor substance arising from platelets. Moreover, as observed in the present studies, perfusion of the ear alternately by defibrinated blood and Ringer-Locke solution showed that flow, though stopped completely, would return to normal repeatedly when the defibrinated blood was washed out of the ear. This could not occur if mechanical block were an important factor.

Constriction of isolated arterial strips or of vessels in situ has been produced by defibrinated blood, by serum (3, 6, 7) and by blood made incoagulable with oxalate, citrate, hirudin (3) or heparin (7). Janeway (3) concluded that uncoagulated blood, while circulating in the intact animal, is not constrictor but that immediately after it is removed from the circulation, and before it becomes thoroughly mixed with the anticoagulant solution, a powerful constrictor substance appears. In agreement with this concept, the perfusion of the rabbit's ear with defibrinated blood produced striking vasoconstriction and unphysiologically slow rates of flow even at high pressures. Page's results, though pressures are not given for individual perfusions, also seem to agree with this conclusion. At perfusion pressures of 140/110 mm. Hg with recirculation the tabulated rates of flow ranged from 18 to 42 drops per minute (6). In a later paper (2) with pressures of 60/40 to 80/60 mm. Hg rates ranged from 9 to 29 drops per minute. In a third series (1) pressure was not specified and the rate of flow ranged from 3 to 36 drops per minute.

For the rabbit's ear simple heparinization of whole blood reduced, but did not abolish uniformly, this progressive vasoconstriction in agreement with Bayliss and Ogden (7) who found "vasotonins" in heparinized blood. Finally with "pre-heparinization" perfusion rates ranged from 8 to 42 drops per minute at pressures of 40/30 to 26/18 mm. Hg. Equally important was the greater constancy of flow in any one preparation over the usual period of one to three hours. If additional donor rabbits were sacrificed, it was possible to continue perfusion for even longer periods without edema and without lessened response to 1:1,000,000 epinephrine. However, since 0.2 cc. of "pre-heparinized" whole blood sometimes produced slight but definite vasoconstriction (table 1) this perfusion medium is still not absolutely physiological, though far superior to defibrinated blood.

It is possible that smaller amounts of heparin would have sufficed but a large excess was used because of the nature of heparin's action (13) and because amounts much greater than those used routinely in perfusion had no effects upon the tone of either dilated or constricted vessels. The minute amount of phenol contained in liquid heparin as a preservative did not affect the results because pure heparin powder (Connaught) dissolved in salt solution and used in similar unitage was quite as effective. Another commercial heparin, Liquaemin-Roche, was tested but its anticoagulant activity per unit volume was far less and its action was correspondingly less dependable.

Dicoumarin[1], or 3,3′-methylenebis-4-hydroxycoumarin, given orally to 10 rabbits for 3 days in doses of 100, 50 and 50 mgm. reduced the vasoconstrictor activity of their shed blood almost as effectively as did "pre-heparinization." It seems likely that the effects of heparin on constrictor activity are due to arrest of coagulation at an early stage, rather than to any specific action of heparin per se.

The minimal constrictor activity of heparinized or "pre-heparinized" plasma and the marked constriction produced by serum differ radically from the results

[1] Dicoumarin was supplied by courtesy of Abbott Laboratories.

of Page (2) who found that serum and plasma of normal or of hypertensive dogs were equally inert when injected into a rabbit's ear perfused with Ringer's solution or with defibrinated blood from a normal dog, while serum and plasma of hypertensive dogs or man were both constrictor when injected into an ear perfused with defibrinated blood from a nephrectomized dog. No explanation of this discrepancy can be offered from our observations.[2]

Preliminary studies in which the rabbit's ear was perfused with "pre-heparinized" rabbit's blood indicate the quantitative significance of constrictor substances from coagulation in assays of circulating constrictor substances by perfusion methods. For a given volume of blood the constrictor activity that appears in the course of coagulation is several times greater than that which can be produced by mixing the same volume of blood with an excess of renin which transforms all the available renin activator into the constrictor substance called angiotonin by Page (1). Under these circumstances it appears extremely important to exclude artefacts due to early coagulation whenever the amount of constrictor substance in the circulating blood is measured by injecting samples of shed blood into an isolated perfused tissue.

CONCLUSIONS

In agreement with previous workers who used other tissues, it was found that defibrinated blood as a perfusion fluid induces marked vasoconstriction in the surviving ear of the rabbit. Heparinized blood was moderately constrictor.

Perfusion of the rabbit's ear could be accomplished with less progressive constriction by injecting heparin into the donor rabbit to arrest coagulation at an early stage before the blood was withdrawn. Precautions against cooling of the ear were also essential for consistent results.

Small amounts, 0.2 cc., of defibrinated blood and serum from normal rabbits, dogs and human beings induce marked constriction. Heparinized or "pre-heparinized" whole blood is much less constrictor but still too variable to be useful in assays.

Plasma rapidly separated from heparinized or "pre-heparinized" blood of normal rabbits, dogs and human beings has little or no vasoconstrictor activity.

These studies emphasize the importance of excluding coagulation when the rabbit's ear method is used to detect constrictor substances of renal or other origin in the circulating blood.

For technical assistance we are indebted to Mr. Herman Goslyn and to Mr. J. E. Wood, III.

[2] Page (14) in a letter dated January 7, 1943 enclosed the following comment to be added as a footnote: "Our method was originally designed to avoid the occurrence of vasoconstrictor substances resulting from coagulation of the blood. Hence my statement in a recent article (2) that serum and plasma can be used interchangeably is completely erroneous. This is the only time that the error has occurred so far as I can find. I confess that I am as puzzled as Dr. Landis as to why the mistake occurred and can account for it only on the basis of a careless misstatement."

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PATTERN OF NORMAL WATER DRINKING IN DOGS

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Intake of water is a discontinuous process; in contrast to output of water which is continuous, and ordinarily almost constant in rate from minute to minute. The discontinuity suggests that the water content of the body periodically becomes sensibly unbalanced, whereas the act of water drinking restores the body and relieves at once the maladjustment of content. What sort of signal initiates water drinking in hourly life? How regular are the periods at which it operates? What sorts of factors modify the periods?

One of nine trained dogs was put in a stall suspended from a Sauter balance. Most observations were made upon two females with exteriorized bladders. In periods of 0.25 hour the insensible loss of weight was ascertained. Urinary loss was found by measuring the urine collected from the exteriorized bladder each 0.25 hour. In most periods, drinking water was available in a can just in front of the dog. Whenever the dog took water, the can and its contents were reweighed. Room temperatures lay between 22° and 26°C. and relative humidities between 30 and 50 per cent.

In some tests the dog did not merely stand in the stall loosely confined, but heat was applied or food was given. Heat was added to the body by two radiators placed laterally at constant position and intensity. The intensity was chosen such that the dog did not fidget. Food consisted of dried whole milk and fox chow, usually mixed with an equal weight of water. The dog swallowed at once all of the food offered. On most days the food allowed each 24 hours was constant, the amount being sufficient for maintenance of body weight.

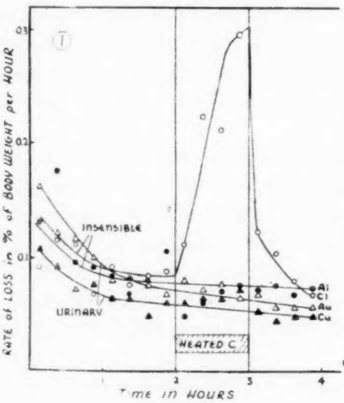
The Sauter balance was used in such a manner that exact equilibrium between its two sides was not required. One minute before the dog's weight was to be ascertained, the balance was allowed to swing, with approximately correct weights in the pan. The midpoints of the free swings just before and just after zero time were averaged; and since the deflection per gram had been previously ascertained with the same dead loads on the balance, the weights in the pan could be corrected, to give an accuracy of weighing of 0.2 gram. Actually only the nearest 0.5 gram differences were ascertained, since these gave 6 per cent accuracy per 1-hour period at the slowest rate of insensible loss.

In each test the dog had been fed 16 hours previously and had been allowed water *ad libitum*. In addition, the dog was offered water upon coming to the stall; if it drank some, drinking to satisfaction was allowed and the test was started; but if it drank none, the dog was not considered to be in a reproducible state of water balance and no test was run upon it that day.

A. *Control*. When water was continuously available, rates of water loss be-

came stabilized about 1 hour after the dog was first weighed (fig. 1). In this period the animal "calmed down" and slight diuresis resulted from the water spontaneously drunk at zero time.

Water was voluntarily drunk in only one instance during ten control tests that lasted 2 hours, in which time 0.35 per cent of the body weight (B_0) had been lost (A, fig. 2). In the one instance 0.29 per cent of B_0 was drunk at 1.6 hours, just restoring the body weight. In two tests that lasted 4 hours, water was taken once at 3.7 hours to the amount of 0.40 per cent of B_0. During the 4 hours 0.60 per cent of B_0 had been lost.



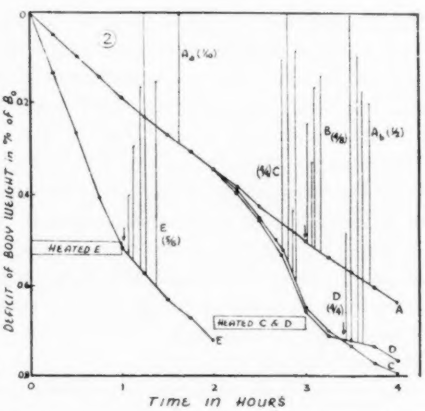


Fig. 1. Rates of water loss from a dog standing in stall. *A*, mean of two control tests. *C*, mean of three tests in which radiant heat was applied for 1 hour.

Fig. 2. Deficits of body weight of dogs, in per cent of initial weight, in various tests mentioned in the text. Vertical lines indicate water voluntarily drunk. In tests *A* and *C* water was always available; in other tests it was allowed after the arrows. A_a, control for 2 hours; A_b, control for 4 hours. *B*, water not allowed for 3 hours. *C*, control for 2 hours, then heated for 1 hour. *D*, control for 2 hours, then water not allowed for 1½ hours while heated in third hour. *E*, water not allowed for 1 hour while heated. Fractions, 1/10, 5/6, etc., indicate number of tests in which water was drunk out of the total tests.

The pattern of ordinary drinking is plain. The dog does not sip water at frequent intervals even though the water is immediately available, but waits some hours between drafts. The draft when taken is not sufficient to restore body weight, but may be considered sufficient to restore water content relative to the body's content of other substances.

B. *No water; later allowed.* When water was not allowed during the first 3 hours, weight was lost as before. Then water was placed before the dog, and in four of eight tests water was drunk within a few minutes. The amounts ingested varied only between 0.18 and 0.38 per cent of B_0, 0.50 per cent of B_0 having been lost. Comparison with the previous result possibly indicates that lack of availability of water tends to induce drinking more frequently when later the water is allowed. But in no case is the amount drunk sufficient to restore body weight.

On the average a loss of weight equal to 0.5 per cent of B_0 is required before drinking supervenes.

C. *Control, then heat.* In these four tests, water was available at all times. After 2 hours of control conditions, heat was applied for 1 hour, the rate of weight loss being greatly accelerated. In the last half of this hour, water was drunk in every instance, and in amounts not sufficient to restore the body weight. Again, 0.5 per cent of B_0 had been lost before drinking occurred (C, fig. 2).

Rectal temperatures fell during the control hours, rose about 0.8°C. during the heating, and fell during 1.3 hours thereafter. The drinking was not clearly related to any particular rise of body temperature.

D. *Heat; water later.* The same form of test was modified by denying the dog water during the heating and for 0.5 hour thereafter. Would the fact that heat no longer impinged on the body lessen the urge to take water? The answer was that just as much water was drunk as in C, in the four tests. Therefore, the heat does not exert its influence only immediately, but also latently. Altogether, heating was scarcely more efficacious than non-heating in induction of water ingestion, at the same deficit of body weight.

E. *Initial heat; water later.* Water was denied after the initial drink of the day. Without a previous control period, heat was applied for one hour (E, fig. 2). Then water was allowed, and in five of six tests was drunk within 0.5 hour. In this series 0.5 per cent of B_0 had been lost in 1 hour instead of 3 hours; no more water was taken here than there. It appears that time is a negligible variable; the amount of water missing from the body is the factor in common.

Summary. Dogs placed under control conditions with minimal physical activities, gradually lost weight by evaporation and by urine formation. Periodically these losses were partially made up by drinking. Drinking rarely occurred before 0.5 per cent of B_0 had been lost, and was insufficient to restore body weight. It occurred somewhat more surely when water had been previously denied the dog, and when the loss had been hastened by heating. The heating may have been over and gone before water was offered, yet drinking occurred more freely. The chief factor in inducing drinking appears to be the lack of body water and not the time that intervened during its loss.

F. *Control with food.* It is shown above that dogs which were fed many hours ago drink little water. This fact has long been recognized in that dogs drink only $\frac{1}{10}$ to $\frac{1}{3}$ as much water upon days when food is withheld as upon days of usual feeding.

In order to study the effects of eating upon drinking, food as well as water was given *ad libitum* at all hours. A popular notion is that dogs cannot be permitted unlimited food; the regime, however, proved satisfactory. The dogs ate somewhat more than was required for maintenance of weight, gaining about 0.5 per cent of B_0 per day on the average.

In these tests (32 days) two dogs remained in metabolism cages. All food and water present were automatically registered. Water level in the drinking pan was recorded as previously described (Adolph, 1939). Dry food was kept in a pan suspended from a spring within the dog's cage; a string from the pan to a

lever indicated the length of the spring and consequently the weight of the food remaining.

The food was taken in about five meals per day. No fixed hours were evident for these meals; but more than average occurred in late afternoon when the dogs were accustomed to being fed, and very few occurred late at night. Only rarely was food merely tasted; instead, eating continued for 5 minutes or more and a mode of 0.18 per cent of B_0 was swallowed at each occasion. Water was rarely drunk between bites, but usually was taken only after eating was finished, when modally 0.38 per cent of B_0 was ingested within 10 minutes. The pattern of water drinking therefore shadowed that of eating. Body weight was fully restored at most meal times. Hence, the periodic taking of food with water eliminated the trend of decrease in body weight that prevailed in the absence of food. Instead, ingestions, which voluntarily occurred every 4 to 6 hours, restored body weight and made it oscillate about a gradual upward trend.

G. *Food at stated intervals.* The relation of food intake to drinking was studied further by allowing portions of food at fixed intervals. Whereas the

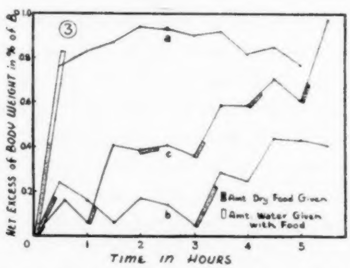


Fig. 3. Excesses of body weight of dogs, in per cent of initial weight, in tests G in which food was periodically given. The weight of food allowed is indicated by thickened lines; all other gains of weight were due to ingestion of water while losses were simultaneously proceeding at 0.08 to 0.20 per cent of B_0 per hour.

whole amount of food had been given (in parts A to E) at 4 p.m., the amount was now divided into portions proportional to the period of time before the next meal.

(a) The dog was kept in the stall for 6 hours, having received $\frac{3}{4}$ of the ration 18 hours previously. In four tests the remaining $\frac{1}{4}$ of the food was mixed with an equal weight of water and given at once. On the average the dog drank water in each subsequent half-hour, and body weight increased further for 2 hours and then gradually declined (*a*, fig. 3). During the decline water was occasionally drunk in each test, but not in amounts sufficient to compensate the simultaneous losses.

This experiment indicates that the periodicities of body weight were chiefly controlled by the periodicities with which food was spontaneously taken by the dog. Although some water was drunk without food, more water was taken at each draft when food had preceded it.

(b) In three tests, $\frac{1}{8}$ of the food was given dry at the beginning of the test, and $\frac{1}{8}$ of it 3 hours later. Body weight increased with each meal, partly because water was ingested soon after it. Thus body weight oscillated in shorter periods.

(c) Finally, in four tests, $\frac{1}{24}$ of the dry food was given each hour for 6 hours. Water was then drunk every hour, usually within 5 minutes after food was swallowed, and correspondingly body weight steadily increased (*c*, fig. 3). Somewhat more urine was produced in these tests.

Summary. It is evident that the pattern of food ingestion may dictate the pattern of water ingestion. The periods between water ingestion could be rendered so small that body weight increased steadily rather than in 6-hourly or in 24-hourly periods. Food ingestion obviated the rule that loss of weight preceded drinking. Rather, the proportion of solids to water within the body was modified by addition of the food instead of depletion of the water.

COMMENT. Drinking is a means of periodically restoring body water content. Previously (Adolph, 1939) it was found that the amounts drunk by the dog were equal to the amounts of water earlier lost from the body during water privation (which could be induced in one to several days while dry food was eaten). Now it is found that the smallest amount of water loss to which the dog usually responds is 0.5 per cent of the body weight. Not the time elapsed (which could be much reduced by heating the dog), but the shortage of water itself appears to set off ordinary drinking. And not the absolute amount of water but the decrement in the proportion of water to other bodily constituents (which could be varied by feeding the dog) seems to arouse the drinking.

These relations naturally result in the observed pattern of water ingestion of the dog. Ordinarily processes of anabolism and catabolism are producing slow changes in the proportions of bodily constituents. Whenever water is out of ratio by about −0.5 per cent of its usual content, a draft of it is taken, in approximately that amount. Usually less water is taken than has been lost since the last drink or meal.

This generalization pictures water content as continually in deficit. But since there is no base line from which to judge deficit other than the usual content of water in the body, it is merely for convenience of reference that water balance in these tests was considered to lie at the body weight of the dog that had just drunk water *ad libitum*. Obviously, too, body weight is but a rough criterion of water balance, since addition to the body of food and water in particular proportions increases the weight without destroying the immediate balance.

It was found that water was usually drunk a few minutes after dry food was eaten. Since the reverse rarely occurred, some support could be deduced for the notion that eating sets off drinking. If so, what releases the act of eating? With food continuously available, some cumulative influence periodically takes effect. To call that influence either hunger or appetite does not help to understand it. It is related to certain or all of the bodily deficits that gradually develop to some critical value before the trigger to eating is released.

The important point here is, however, that if the dog's five meals a day are stopped by deprivation of food, drinking that ordinarily follows each meal goes on just the same. The amounts drunk are reduced, but the same number of drafts are taken per day. Hence the pattern of drinking is demonstrated to exist independently of eating, though ordinarily the two work together in one sequence. The fact that some water is drunk during the deprivation of food

may indicate that water lost by the quiet dog is in excess of the body water liberated from its connection with catabolized constituents of the body. The constancy of urinary composition opposes the view that much water is drunk in excess of needs under any circumstances.

While the above rules held for the dogs studied in the laboratory, it is quite probable that deviations from these rules with respect to certain details will be found in other individuals, and particularly in individuals not conditioned to laboratory existence.

The sensitivity of the dog to the deficit of water which leads to drinking is such that on the average the dog drinks when 0.5 per cent of the body weight has been lost. Whatever sense organs or nerves are sensitive to want of water, and initiate sensations of thirst, themselves set in motion the response of drinking at this deficit of water. The result is an oscillation of water content of the body through sudden periodic intakes. But if food were supplied continuously at a rate equal to catabolism, the fluctuations to either side of the mean would be expected to amount to only 0.25 per cent of the body weight.

It is noted that the dog does not take its water in as continuously as the frog does. No terrestrial animal does so. It is appropriate to point out that intermittency of an activity allows successions of activities that cannot be carried on simultaneously. With respect to water, the dog is able to spend 99 per cent of its time away from water, providing that water can be reached at appropriate intervals. An animal as large as the dog is able, in absence of marked physical activity, to be away from water for 4 to 6 hours without change of more than 1 per cent in its water content. Hence body fluids and cells are kept approximately that constant in composition in spite of the intermittent character of intakes of water and food.

The picture that we derive from the above observations is that 1, the state of water balance is continually changing in relation to body weight, according as other metabolic processes are active; 2, change in rates of catabolism or sudden addition of food or solute to the body shifts the state of water balance accordingly; 3, the actual content oscillates about the virtual content at which water is balanced; 4, the period of this oscillation varies with rates of water exchange; but 5, the amplitude of this oscillation is ordinarily quite constant, through the fact that the dog drinks whenever the deficit of water is great enough to arouse an effective thirst. Many details remain to be added to this general statement.

SUMMARY

The conditions of water loss and of food gain were varied while spontaneous drinking was observed in dogs. The signal that initiates water drinking in ordinary life appears to be a deficit of water relative to other bodily components. Whenever the body is depleted of water by about 0.5 per cent of the body weight, water is drunk. The amount drunk is accurately proportioned to the body's water deficit at each draft, though no absorption of water has time to occur before drinking ceases. Body weight is only a rough criterion of water balance, even under restricted circumstances, since addition of food and loss of catabolic products complicate its relations.

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THE ACTION OF ADRENALINE, ACETYLCHOLINE AND POTASSIUM IN RELATION TO THE INNERVATION OF THE ISOLATED AURICLE OF THE SPINY DOGFISH (SQUALUS ACANTHIAS)[1]

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It seems fairly well established that the elasmobranch heart is not supplied with sympathetic nerve fibers. Lutz (1930a) has reviewed and confirmed the evidence for this view. Since the heart appears to be exclusively under vagus control, the isolated sinus-auricle preparation seemed a good one for the investigation of the action of adrenaline on myocardial tissue lacking sympathetic innervation. MacDonald (1925) and Lutz (1930b) have found adrenaline to have a vagus-like inhibitory effect on the heart beat of elasmobranchs, often followed by a stimulating action. However, this effect was with concentrations of adrenaline (1:25,000–1:100,000) unlikely to exist under physiological conditions in this animal's heart, particularly since, if there is no sympathetic supply, only the adrenaline in the circulating blood is available to affect the myocardium. In our reinvestigation of the effect of adrenaline we have used weaker and hence more nearly physiological concentrations. The study of the action of adrenaline has also led to observations on the actions of acetylcholine and potassium-excess.

Methods. The sinus-auricle preparation of the spiny dogfish (Squalus acanthias) was suspended in a cylinder of 50 cc. capacity containing the physiological saline for elasmobranchs described by Lutz (1930b) and so arranged that contractions were recorded on a kymograph. A stream of oxygen was bubbled through the solution during the whole period of observation. Auricles continue to beat under these conditions for at least 24 hours.

Drugs were added in solution to the cup where they were quickly dispersed by the stirring action of the oxygen bubbles.

Results. We were able to confirm the observation of MacDonald and Lutz that adrenaline in quite large concentrations (1:2000–1:100,000) will cause a temporary inhibition of the heart followed by a stimulation. In contrast to this finding, dilute solutions of adrenaline (1:1,000,000–1:200,000,000) caused only stimulation, which was recorded as an increase in the amplitude of the contractions without much change in rate. Neither of these effects of adrenaline could be blocked by atropine, which is evidence against the hypothesis of Lutz that the inhibitory action of adrenaline observed by him was due to stimulation of parasympathetic nerve endings.

In the absence of an inhibitor of choline-esterase (e.g., eserine), concentrations of acetylcholine as high as 1:5000 were required to produce any inhibitory effect. This is in marked contrast to the reaction of the auricles of frog and turtle hearts,

[1] Aided by a grant from the Bristol-Myers Co.

which under the same conditions show inhibitory effects when exposed to acetylcholine in concentrations a thousand times more dilute. The implication of this observation is that the choline esterase content of the tissue is quite high. This inference is strengthened by the observation that the auricles show a decreased amplitude of contractions in an acetylcholine concentration of 1:100,000,000 after treatment with prostigmine (1:500,000) (fig. 1), although much higher concentrations were required to cause complete suppression of the beat (fig. 2). The reaction of this auricle to acetylcholine is like that of other vertebrate auricles in that small concentrations cause only a negative inotropic effect whereas greater concentrations are required to cause negative chronotropic effects which eventually lead to complete cessation of contraction.

Potassium excess also suppresses the contractions of this auricle. An excess of 30 mgm. per cent of KCl (in addition to the 60 mgm. per cent already present in

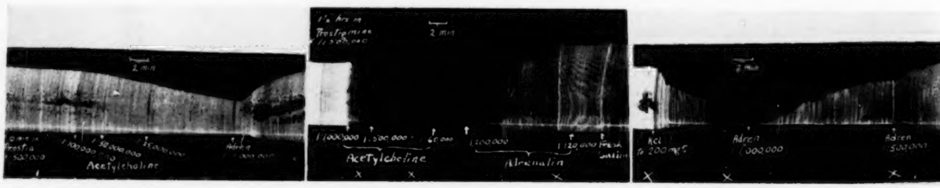

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Effect of acetylcholine (1:100,000,000 to 1:25,000,000) after prostigmine (1:500,000) on the isolated sinus-auricle of Squalus acanthias, and its reversal by adrenaline (1:1,000,000).

Fig. 2. Effect of gradually increasing concentrations of acetylcholine (1:1,000,000 to 1:60,000) after prostigmine (1:500,000) on the isolated sinus-auricle of Squalus acanthias, and its antagonism by adrenaline (1:1,200,000 and 1:120,000). Sections of the original record have been omitted at the points marked X.

Fig. 3. Effect of potassium excess (200 mgm. per cent KCl) on the isolated sinus-auricle of Squalus acanthias and its antagonism by adrenaline (1:1,000,000 and 1:500,000). Sections of the original record have been omitted at the points marked X.

the normal saline) caused a decrease in the amplitude of the contractions, and an excess of KCl up to 200–300 mgm. per cent caused complete cessation of the beat (fig. 3). This action of potassium is not blocked by atropine and the evidence seems conclusive that its effect is directly on the auricular myocardial cells, its action being identical with that which has been demonstrated in the case of the non-innervated turtle ventricle by Hiatt and Garrey (1943).

It is significant that weak concentrations of adrenaline antagonized not only the neural inhibitory action of acetylcholine but also the myal depression produced by potassium-excess. After the auricle has been inhibited to the point of complete cessation of contraction by either of these agents, the addition of small quantities of adrenaline causes a resumption of the beat and a recovery of the original amplitude in the presence of the inhibiting or depressing substance (figs. 2 and 3). The corresponding antagonisms found in the auricles of frog or

turtle hearts do not approach the degree noted in these experiments on the dogfish heart.

Discussion. If the elasmobranch heart lacks a sympathetic nerve supply, and this seems well established, then the site of action of adrenaline must be either on the vagus endings or directly on the myocardial cells. The fact that adrenaline effects, both augmentor and inhibitory, are unaffected by atropine would appear to indicate that the action is not on the vagus endings. Therefore the site of action of adrenaline, including the antagonism of the effects of acetylcholine and potassium-excess, must be directly on the myocardial cell. Hiatt and Garrey (1943) have shown that adrenaline has a slight augmentor action on the contractions of spontaneously beating strips of turtle ventricle, which they believe to be without autonomic innervation. The effect of adrenaline on the myocardium of the elasmobranch auricle is, however, much greater, and it would appear that the myocardial cells of the elasmobranch auricle have a special sensitivity to adrenaline.

The heart of the elasmobranch is apparently under constant vagal tone and since stimuli applied to almost any part of the body of an elasmobranch cause reflex cardiac inhibition (Lutz, 1930a), it is apparent the extreme sensitivity of the myocardium to adrenaline affords a mechanism of antagonism to acetylcholine even in the absence of sympathetic innervation of the heart. It has been shown by Lutz and Wyman (1927) that extracts of the chromophil bodies of this elasmobranch give characteristic adrenaline effects. Whether these glands are activated in an emergency is not yet known.

SUMMARY

1. The isolated sinus-auricle preparation of the elasmobranch (Squalus acanthias) which is apparently without sympathetic innervation, shows marked reactions to adrenaline.

2. The previously reported observation that adrenaline in high concentration causes a transient inhibition has been confirmed, but this effect is not blocked by atropine, so it is concluded that the action is not upon the vagus endings as has been suggested, but that it is directly upon the myocardial cells.

3. Adrenaline in low, more nearly physiological concentrations causes augmentation of the contractions without a change in rate. This effect also persists after atropine.

4. The dogfish auricle is much more resistant to the action of acetylcholine in the absence of an inhibitor of choline-esterase than the auricles of frog and turtle hearts, but after treatment with prostigmine the dogfish auricle reacts in the same manner and with approximately the same sensitivity as other vertebrate auricles to acetylcholine.

5. The inhibitory actions of acetylcholine and the depression of auricular beat due to potassium-excess are markedly antagonized by small concentrations of adrenaline.

6. A possible teleological value of the sensitivity of the myocardial cells to adrenaline is suggested.

The author wishes to express his gratitude to Dr. W. E. Garrey for his criticism during the writing of this paper.

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LOW HEART RATE IN THE NEWBORN RAT

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Curiosity about whether the heart rate of the one-day-old rat could be electrically recorded gave rise to the following series of experiments. It was found that heart rate could be recorded[1] and that, contrary to expectation, the rate of the day-old rat was considerably lower than that of the adult rat. The present series of experiments was planned to verify this surprising initial finding and to study the development of heart rate in the early stages of the rat's life.

Determination of heart rate in newborn rats. The heart beats were recorded by a Davis electroencephalograph; clip electrodes were attached ventrally and dorsally through the axis of the heart.

Heart rates were determined in 131 rats from 19 litters within 24 hours of birth. The rate per minute of each animal was computed from a 30-second strip of electroencephalograph tape. Fluctuation in heart rate was calculated by analyzing the 30-second period into five 6-second periods, and considering the maximum change between any two periods as the fluctuation. Data were analyzed for sex differences, and comparison with mature, 120-day-old rats was made.

RESULTS. *a.* A comparatively low heart rate was found in both male and female day-old animals. Sex differences in mean heart rate found in the tamed adults were not present in the newborn animals. (A slight but significant difference in body weight was found in favor of the males.) No sex differences in fluctuation of heart rate occur.

b. Significant differences in heart rate between male and female 1-day-old and 120-day-old rats were found. The 1-day-old rats had a much slower rate. The range of individual differences in mean heart rate (as indicated by "V", the coefficient of variability) decreases from the 1-day level to the 120-day level. Average fluctuation in heart rate increases significantly from the 1-day to the 120-day-old animals. Individual differences in average fluctuation (as indicated by "V") decrease from the 1-day to the 120-day-old animals.

c. Of the 131 rats, 99 (from 14 litters) had been tested immediately following birth, while 32 rats (from 5 litters) had been tested from 8 to 24 hours after birth. Data were re-analyzed to see whether heart rate differed between these two age groups.

Significant changes in heart rate occurred within the first 24 hours of life. No significant differences in mean fluctuation of heart rate were observed in the first 24 hours of life. Sex differences in mean heart rate were not present at birth or in the first 24 hours of life.

d. In the above analysis significant changes in heart rate were found in dif-

[1] A check experiment in which the thoracic cavity was opened and the heart observed simultaneously with the recording ensures that it was the heart potential which was being recorded.

ferent rats during the first 24 hours of life. The aim of the present experiment was to follow the changes in heart rate in the same animals through the first 24 hours of life. To this end the following procedure was adopted: 12 animals,

TABLE 1

Mean heart rate and mean fluctuation in heart rate in a 30-second period of 131 rats during the first 24 hours of life

	MALES (N:66)	FEMALES (N:65)	CRITICAL RATIO*
Mean heart rate.....	174.6 ±44	168.5 ±42.7	0.8
Mean fluctuation of heart rate.	12.7 + 14.3	13.1 + 16.4	0.2

* This is an index of the reliability of a difference between two measures. A critical ratio of 3 or more indicates that the obtained difference is statistically significant, *i.e.*, the chances are greater than 999 in 1000 that a true difference exists.

TABLE 2

Comparison of mean heart rate and mean fluctuation in heart rate in 1-day-old and 120-day-old (tamed)‡ rats

	1-DAY-OLD GROUP		120-DAY-OLD GROUP		CRITICAL RATIO COLUMNS (1) & (3)
	Mean (1)	"V"† (2)	Mean (3)	"V"† (4)	
Male rate......	175 ±44 (N = 66)	25.1 (23.8)*	433.5 ±22 (N = 16)	5	34.1
Fluctuation.....	12.7 ±14.3 (N = 66)	113 (116)*	22.3 ±8.2 (N = 16)	37	3.7
Female rate.....	168.5 ±43 (N = 65)	25.6 (25.5)*	476 ±24.6 (N = 11)	5	33.4
Fluctuation.....	13.1 ±16.4 (N = 65)	129 (133)*	19.5 ±3.6 (N = 11)	19	2.9

* Since it will be shown that heart rate varies significantly in the first 24 hours of life, the objection might be raised that considerable variability in the rate of the 1-day-old rats is due to the presence of different age groups (in terms of hours). To meet this objection, results were tabulated for 50 males and 49 females measured directly at birth. These results, not significantly different from those for the total group, are given in parentheses.

† Coefficient of variability.

‡ These animals had been habituated to the heart recording situation for a 2-week period before records were taken.

TABLE 3

Comparison of mean heart rate and fluctuations in heart rate of rats tested at birth and of rats tested 8 to 24 hours after birth

INDEX	GROUP TESTED AT BIRTH (N:99) (1)	GROUP TESTED 8-24 HOURS AFTER BIRTH (N:32) (2)	CRITICAL RATIO COLUMNS (1) & (2)
Mean heart rate...	161 ±40 (CR* sex diff: .6)	204.2 ±40 (CR* sex diff: .6)	5.3
Mean fluctuation in heart rate	14 ±18.4 (CR* sex diff: .2)	9.2 ±11.8 (CR* sex diff: .1)	1.6

* Critical ratio.

TABLE 4

Significance of differences in heart rate between 8-hour periods in the first 24 hours of life

PERIOD	MEAN HEART RATE (N:12)	CRITICAL RATIO OF SUCCESSIVE PERIODS
Birth....	172 ±14	
8 hrs.......	211.3 ±16.8	6.3
16 hrs........	225.5 ±23	1.7
24 hrs........	257.3 ±23.6	3.4
Birth-24 hrs.		10.8

6 males and 6 females, from two litters, were tested immediately at birth and every 8 hours thereafter for the first 24 hours of life.

Results on the same animals confirm what had previously been found in

different animals, *i.e.*, significant changes in heart rate occur in the first 24 hours of life. The more detailed analysis in this experiment showed that within the first 8 hours of life significant changes are to be noted.

e. As a continuation of preceding experiments a study of changes in heart rate in the first 21 days of life was made. Seventeen rats, 10 males and 7 females, were tested daily at the same hour.

Mean daily heart rate over the 21-day period was computed for both sexes.

Increase in heart rate was greatest in the first 11 days of life. By this time the rates of both males and females exceed 400 and overlap at times with the range found for the tamed 120-day-old rats. From the 11th to the 21st day a plateau of little or no increase in heart rate occurs. Sex differences emerge more definitely from the 10th day of life on, the females having the higher rate. However, sex differences in heart rate in mature rats were found to exist only in tamed animals habituated to the recording system. Untamed mature animals showed no sex differences, nor did the tamed animals when recording was resumed after a 50 day interval. Evidence on hand would suggest an interpretation in terms of the differential reaction of the male and female heart under conditions of stress, the male rate increasing to a greater degree than the female and in this way reaching a level not significantly different from the females.

Discussion. In the foregoing experiments the most striking result concerns the comparatively low heart rate of the 1-day-old rat. The expectation had been that a rate of 600–800 beats per minute would be found at birth with a subsequent decrease to the adult level. Whether this low rate at birth is peculiar to the rat alone is not definitely known, for there has been little comparative work done in this respect. A few exploratory experiments carried out by the writers with newborn kittens and pigs showed the usual result, *i.e.*, a heart rate at birth higher than that of the adult. The mean heart rate of two newborn kittens was 168, while that of two adult cats was 131. In like fashion the mean heart rate of two newborn pigs was 227, and that of three adult pigs, 65. Similar results with other animals are reported by Dukes[2] and he concludes: "Young animals have a faster heart rate than mature animals . . . explained at least in part by their smaller size" (p. 115).

SUMMARY

1. One-day-old rats, when compared with 120-day-old (tamed) rats, were found to have: *a*, a significantly lower heart rate; *b*, less fluctuation in heart rate. Sex differences present in the tamed, mature animals were not present in the 1-day-old animals.

2. In the first 24 hours of life significant changes in heart rate were observed.

3. In a 21-day period of daily heart recordings, it was observed that:

a. Heart rate increased steadily in the first 11 days of life.

b. A plateau of no increase in rate prevails from the 11th to 21st days.

c. Sex differences manifest themselves with increasing clarity from the 10th day on.

[2] Dukes, H. H. The physiology of domestic animals. Comstock Press, Ithaca, N. Y., 1942, pp. 721.

DIFFERENTIAL EFFECTS OF STRETCH UPON THE STROKE VOLUMES OF THE RIGHT AND LEFT VENTRICLES

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In cinematographic studies on ventricular output Shuler, Ensor, Gunning, Moss and Johnson (1941) employed a modification of a technique developed in several laboratories by Takeuchi (1925), Strughold (1930), Landis, Hunt, Moe and Visscher (1940), and Burchell and Visscher (1941). Shuler and co-workers differentiated the effects of respiration on the output of the right and left ventricles and found that in each respiratory cycle the stroke volumes of the right and left ventricles varied independently. They reported an *increase* in the stroke volume of the *right* ventricle and a *decrease* in the stroke volume of the *left* ventricle during inspiration with the reverse changes occurring during expiration. These periodic independent fluctuations in output occurring in each ventricle were associated with corresponding independent fluctuations in the diastolic size of the two ventricles such that, when the diastolic size of the right ventricle increased in inspiration the stroke volume of that ventricle also increased, even though the diastolic size and stroke volume of the left ventricle simultaneously decreased.

In view of this evidence that each ventricle, independently, "obeys" the "Law of the Heart" (Patterson, Piper and Starling, 1914), which states that "... the energy set free at each contraction of the heart is a simple function of the length of the fibers composing its muscular walls," it was felt that further studies should be made on the independent operation of this law in the two ventricles.

METHODS. Dogs were anesthetized with sodium barbital. A portion of the sternum and ventral chest wall was removed and the pericardium was incised and sutured to the chest wall to cradle the heart. Numerous small circular cardboard markers were fixed to the heart clearly outlining measurable areas on the surface of each ventricle. Motion pictures were taken (at 32 or 48 frames per sec.) through a transparent window used to seal the opening in the chest wall, permitting the dog to breathe in the normal manner. In some experiments, the pictures were taken without sealing the chest wall, exposing the heart to atmospheric pressure.

Various procedures which change the diastolic size and, consequently, the force of the stroke were employed. These included: 1, increasing the venous return by rapid intravenous infusion of Ringer's solution; 2, increasing the peripheral systemic resistance by partially occluding the abdominal aorta, and 3, normal respiratory changes in intrathoracic pressure, pulmonary vascular capacity and ventricular filling.

Negative enlarged prints of many successive frames were made, and the out-

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lined area of each ventricle was measured in each frame. Plotting these areas gave approximations to "volume curves" showing maximal diastolic size, minimal systolic size, and "stroke volume" for each beat of each ventricle, in arbitrary units. For a number of heart beats, diastolic sizes of the right and left ventricles were plotted separately against the corresponding stroke volumes, and differences in the steepness of the slopes of the curves for right and left ventricles were interpreted as differences in the effectiveness of stretch of the muscle fibers of the two ventricles in inducing increases in stroke volume.

RESULTS. Figure 1 shows typical results obtained in most instances in which changes in diastolic size and stroke volume were produced by means of intravenous infusion of Ringer's solution or compression of the aorta while the cardiac rate remained constant. It is seen that the points for the right ventricle are less scattered and fall more nearly upon a line than those for the left ventricle. Prob-

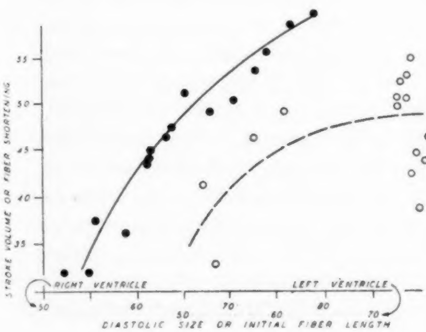

Fig. 1

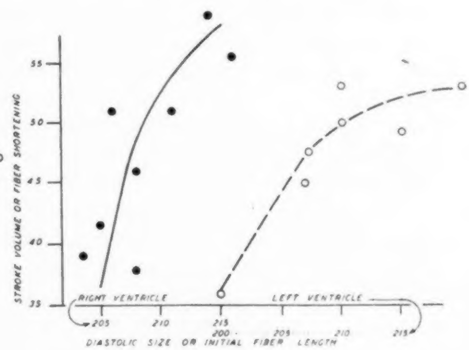

Fig. 2

Fig. 1. Effect of increase in diastolic size induced by intravenous saline infusion and aortic compression upon the stroke volume of the left and right ventricles determined separately. Units are arbitrary.

Fig. 2. Effect of changes in diastolic size induced by normal respiration upon the stroke volume of left and right ventricles determined separately. Units are arbitrary.

ably this indicates that the relationship between diastolic size and stroke volume is more direct in the right ventricle, i.e., it is influenced less by such other factors as changes in peripheral resistance or velocity of ejection than is the case in the left ventricle.

The greater steepness of the curve for the right ventricle seems to indicate that increased diastolic size (and stretch of the muscle fibers) is more effective in increasing the stroke volume of the right than of the left ventricle.

Figure 2 shows eight beats of the heart during a single respiratory cycle, in which the diastolic sizes and stroke volumes of the two ventricles fluctuate independently. The results are essentially the same as in figure 1. In the right ventricle, the points are again somewhat less scattered, and a given increment in fiber length seems to be more effective in increasing the systolic shortening of the fibers in the right than in the left ventricle.

In a few instances, the steepness of the slope of curves for the right and left ventricles was nearly the same, but the steepness of the left ventricular curve was never greater than that of the right ventricle in the same heart.

DISCUSSION. In attempting to account for these differences in response to stretch in the right and left ventricles, it must be borne in mind that in these experiments only *one* manifestation of increased work resulting from increased stretch is measured. For a complete analysis it is necessary to know not only the stroke volume but also the pressure (peripheral resistance) against which each ventricle acts as well as the factor for imparting velocity to the blood. For example, in aortic compression (one procedure providing data for fig. 1) the systemic peripheral resistance was certainly increased. Therefore, much of the extra energy set free by the stretched fibers of the left ventricle was employed to overcome the increased resistance. Consequently, less of the extra energy was available for increasing the stroke volume. On the other hand, compression of the aorta leads to a relatively slow and slight rise in pulmonary pressure (Johnson, Hamilton, Weinstein and Katz, 1937) so that a greater percentage of the extra work done after stretch of the fibers of the right ventricle was employed to produce increased stroke volume. However, when Ringer's solution is infused there is no such marked increase in arterial resistance in the systemic circulation as compared with that of the pulmonary system. Yet, essentially the same differences in the effects of stretch upon the two ventricles were seen.

The results plotted in figure 2 may also be partially explained by fluctuations in the resistance against which each ventricle pumps, in each respiratory cycle. When the diastolic length of the right ventricular fibers is greatest (i.e., in inspiration) the resistance against which that ventricle pumps is lowest, since pulmonary arterial pressure has been shown to fall in inspiration (Johnson, Hamilton, Weinstein and Katz, 1937). Therefore, the added force of contraction produces mainly an increased stroke. On the other hand, when the diastolic length of the left ventricular fibers is greatest (i.e., in expiration) the systemic arterial pressure is highest. Here the added force of contraction can be only partially employed to increase the stroke, since some of the added force must be used to pump against a higher pressure.

Visscher and Starling (1927) have shown that a poorly nourished heart must be stretched more than a well nourished one to induce the same increment of work. It seems unlikely that the left ventricle was regularly less well nourished than the right, in our experiments, since the systemic blood pressure remained at a good level throughout, and the fibers of both ventricles are supplied from the aorta. Furthermore, the left ventricular fibers are stretched the most during expiration at which time their blood supply should be greatest since the aortic pressure is highest at this time.

It seems probable that differences in the anatomical arrangement of the muscle fibers in the two ventricles may be mainly responsible for the results obtained. The muscle fibers are known to run a more nearly straight course in the right than in the left ventricle,[2] so that an increase in the area of an outlined portion

[2] Jane Sands Robb: Personal communication.

of surface of a ventricle represents a greater actual increased initial length of a given fiber in the right ventricle than in the left.

It seems improbable that the properties of the muscle fibers of the two ventricles are inherently sufficiently different to account for the apparently greater effect of stretch upon the stroke of the right than of the left ventricle.

SUMMARY AND CONCLUSIONS

1. Using a cinematographic technique, the effect of varying the diastolic stretch of the cardiac musculature (i.e., initial fiber length) upon the subsequent systolic stroke (i.e., shortening in contraction) was determined in the left and right ventricles of dogs separately.

2. The relationship of initial diastolic fiber length to stroke volume was more direct in the right than in the left ventricle, presumably because the peripheral resistance or pressure factor in the work done by the heart fluctuates less in the pulmonary than in the systemic circuit.

3. A given increment in diastolic size of the right ventricle was found to be more effective in producing an increased stroke volume from that chamber than was the same increment in the left ventricle.

4. Differences in the nourishment or inherent properties of the left and right ventricles are probably not sufficiently great to account for these findings.

5. In some of the procedures employed (especially occlusion of the aorta), the smaller stroke volume increase of the left ventricle in response to stretch may be accounted for partially by a concommitant increase in peripheral systemic resistance. Under such conditions much of the extra energy is employed in overcoming the increased resistance, leaving less energy to increase the stroke volume. Since aortic occlusion does not elevate the pulmonary peripheral resistance appreciably, a larger proportion of the extra energy released by stretch of the right heart is employed in increasing the stroke volume.

6. The most important factor accounting for the apparently greater response of increased diastolic size in the right ventricle is probably the fact that the muscle fibers of the right ventricle pursue a straighter course than the fibers of the left ventricle, so that a given increase in diastolic size produces a greater actual stretch of the muscle fibers themselves in the right than in the left ventricle.

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FACTORS INFLUENCING THE TEMPERATURE REGULATION OF BIRDS[1]

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In his recent paper on the development of body temperature of birds, Kendeigh (1939) recalls the early work of Edwards who in 1894 divided young birds into two categories, those with (precocial) and those without (altricial) temperature regulation at hatching. The chick has been suggested to be of the precocial group. Romanoff and Sochen (1936) and Romanoff (1941) demonstrated that although the chick embryo is poikilothermic during early development, it changes toward the homoiothermic state during incubation and reaches true homoiothermism four or five days after hatching.

The development and importance of shivering and panting in thermotaxis of birds is not well understood. Odum recently introduced a sensitive recording device for recording shivering and found (1942) that muscle tremors of shivering do not appear in small altricial birds until at least three days after hatching. Randall and Hiestand (1939) found that panting in the domestic fowl is roughly proportional to body temperature and that the panting center is relatively inactive at normal temperatures.

In this study experiments were devised to investigate the development of temperature control in the chick, the nature and significance of shivering and panting, the effect of relative humidity on the efficiency of panting in temperature control, and the upper lethal temperature.

Studies were conducted with barred-rock chickens (*Gallus domesticus*) and pigeons (*Columba livia*). Development of temperature control was studied upon chicks removed from the incubator two hours after hatching and at progressively older ages until after thermotaxic control was established. Body temperatures were recorded from thermometers placed to a constant depth in the cloaca, under the skin, and in some instances in the visceral regions. First muscle tremors of shivering were heard through a stethoscope placed upon the muscles of the body.

A temporator tube used in lowering body temperature in some experiments consisted of a large T-tube, one end of which was enlarged into a bulb. A smaller tube was inserted through the distal opening of the T-tube and water was pumped into the bulb and out by way of the side tube maintaining a constant circulation of water at any desired temperature. In other experiments the body temperature was decreased in an ice chamber, or increased by wrapping the body in an electric heating pad. In order to raise skin temperature while

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maintaining a normal deep temperature, the body was wrapped in a heating pad while cold water was circulated through a rubber-lined metal collar placed around the neck.

In studying the effect of humidity in temperature control, the body temperature of pigeons was raised in a heating cabinet devised to allow changes in relative humidity.

EXPERIMENTAL RESULTS. *Development of body temperature control.* When young chicks (2 to 24 hrs. post hatching) were removed from the incubator their body temperatures closely approximated that of the incubator (38 to 39°C), and when exposed to laboratory temperatures of 26°C, their body temperatures rapdily fell as low as 31 to 32°C. Upon exposure to more severe environmental temperatures (ice chamber at 10°C) practically no resistance was shown to the

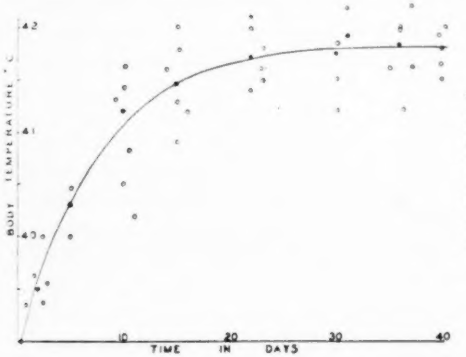

Fig. 1

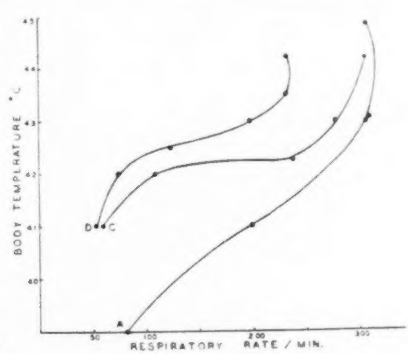

Fig. 2

Fig. 1. The increase in body temperature of chicks from a few hours after hatching to that of chicks 40 days old. The curve represents average temperatures while the circles represent the range of variation. First two averages were taken with the birds in an environmental temperature of 38 to 39°C.; remainder at 20 to 26°C. This progressive development was studied in 40 birds.

Fig. 2. Panting responses in young chicks as determined at different ages. Curve *A* is that of a two-day-old chick. *C* is that of the same chick fifteen days old and *D* is that of the chick twenty-eight days old.

chilling. Shivering was not pronounced and was often absent. The seven-day-old chick responded with vigorous shivering when exposed to the laboratory temperature and was completely capable of maintaining its body temperature within normal levels of 40.5 to 41°C. In fact, thermogenesis was sometimes sufficient to raise body temperature a few tenths of a degree. Exposure to temperatures below 20° still caused a rapid fall in temperature however and protection against such environmental temperatures did not develop until the down feathers were well replaced by adult plumage.

Two days after hatching the body temperature was above that of the incubator and a continued average rise was recorded until about 22 days after hatching (fig. 1). The most rapid increase occurred in the first ten days and there was but little change except for the usual diurnal variations after 15 days.

Newly hatched birds were found to be fully capable of responding to increased body temperatures by polypneic breathing which quickly assumed the characteristics of panting, but the panting was initiated at a lower level than in adults (table 1).

The gradual rise of the panting threshold parallels the rise of normal body temperature, but since it does not rise as rapidly, there results a progressive decrease in the thermal tolerance. Thus the thermal tolerance gradually approaches that of the adult, the panting threshold becomes sharper and the efficiency of the panting mechanism increases (fig. 2).

TABLE 1

Panting responses of a typical chick during development from 1 day to 42 days of age

This chick was chosen as typical of 20 birds

AGE	CLOACAL TEMPERATURE	PANTING THRESHOLD	THERMAL TOLERANCE
Days	°C.		
1	38.0	41.0	3.0
5	40.0	42.0	2.0
10	40.8	42.1	1.4
15	41.4	42.5	1.1
22	41.7	42.8	1.1
42	41.7	43.0	1.3

TABLE 2

A condensed protocol chosen as typical of responses of the week-old chick to decreasing body temperature

Thirty birds were used

TIME	CLOACAL TEMPERATURE	SKIN TEMPERATURE	HEART RATE PER MINUTE	RESPIRATORY RATE PER MINUTE	REMARKS
Minutes	°C.	°C.			
0	42.0	41.8	300	50	
2	41.9	41.0	360	70	First tremors
10	41.3	40.0	360	80	Continuous shivering
30	38.0	35.0	320	80	
52	35.0	31.0	270	76	
75	31.0	28.0	200	52	
110	26.0	23.0	108	40	Shivering decreasing
130	23.0	20.0	52	24	
150	20.0	18.0	20	14	Shivering ceased
180	15.0	13.0	12	0	

Shivering and falling body temperature. The first muscle tremors preceding shivering in the seven-day chick (in ice chamber at 10°C) were noted after a fall of 1 to 3°C skin temperature with little or no change in cloacal temperature. Vasoconstriction and periodic muscle tremors were quickly followed by continuous shivering and in all instances were accompanied by faster breathing and heart rates (table 2). With progressively falling body temperatures shivering began to decrease in intensity at about 26°C and ceased entirely at about 20°C.

Respiration failed at 15°C and although the heart became very weak, biopsy showed that it continued to beat at temperatures as low as 8.5°C. Administration of 100 per cent oxygen into the trachea and out by way of a punctured abdominal airsac maintained a respiratory rhythm (6 per min.) at a temperature of 10°C indicating that hypothermic death is primarily caused by anoxic paralysis of the respiratory mechanism. Complete recovery was brought about if body temperature was returned to normal in a heating pad providing cloacal temperature was not reduced previously below 15°C.

To further investigate the existence of reflex and central shivering, a temperator tube was placed in the cloaca and body temperature was lowered without subjecting the skin to sudden changes in temperature. In contrast to the above experiments, shivering first appeared only after a fall of 1.2°C cloacal temperature while the skin temperature was held relatively constant. It therefore appears that shivering may be produced reflexly by stimulation of cold receptors in the skin or centrally by cooling effects of the blood bathing thermogenic centers.

Panting and rising body temperature. In contrast to experiments upon mammals, reflex panting of birds has not been demonstrated. An experiment was therefore devised whereby skin temperature was raised while at the same time the carotid blood was maintained at or below normal temperature. This was accomplished by placing the bird in a heating pad while the metal collar with circulating cold water was in place around the neck.

With this technique it was possible to maintain the deep body temperature at a constant level for two hours even though the skin temperature was raised nearly four degrees. This indicates the cooling effect of the collar was sufficient to chill the blood enough to offset the warming of the body through the skin. Breathing remained normal in rate and amplitude. The temperature of the esophagus under the collar was 37.6° while that of the skin under the heating pad was 45.0°C. Upon removal of the collar, with heating pad still in place, the breathing rate increased instantly from 34 to 150 per minute (table 3). Thus even though the temperature of the skin was held at or above panting levels for 2 hours, panting did not occur until the thermoregulatory centers were warmed to panting levels by the circulating blood. This indicates a discrete central control of panting in the fowl.

Effects of varying humidity upon panting. Since birds have no sweat glands and since feathers prevent much evaporation from the skin, the burden of heat loss must fall upon respiration. It is therefore important to know the effects of varying humidity upon the ability of birds to adequately control body temperature (table 4). The panting threshold was not markedly changed when both environmental temperature and relative humidity were increased, nor was thermal tolerance significantly altered. The bird's ability to prevent a rapid rise in body temperature was greatly inhibited, however. The time required to reach the panting threshold was decreased and the efficiency of the panting mechanism was decidedly less than normal, due to decreased evaporation of water.

The upper lethal temperature of birds. Available literature has revealed no definite information concerning the physiological effects of prolonged and acute hyperthermia. After reaching the polypneic stage of panting, with continuous hyperthermia, respiratory failure was one of the first indications of physiological breakdown. This followed a preliminary slowing from the maximum breathing rate and occurred at a temperature above 45.5°C. As cloacal temperature approached 47.0°, rising more rapidly after failure of the panting mechanism, the heart began to decrease in rate. Breathing was reduced to a few dyspneic gasps interspaced by apnea. Death was always immediately preceded by increasing tonus of skeletal muscles which reached convulsive stages just prior to complete respiratory failure. The convulsions were characterized by a "stretching" reaction similar to those noted in asphyxial death. These convulsions then were replaced by decreasing clonic contractions; the heart became very irregular and weak. Immediate autopsy showed that the heart had completely stopped except for signs of flutter or fibrillation. All of these later changes were noted within a period of one to three minutes before death.

TABLE 3

A condensed protocol showing respiratory responses produced by rising skin temperatures while blood flowing to the brain is cooled

TIME	CLOACAL TEMPERATURE	SKIN TEMPERATURE	HEATING PAD	COLLAR	RESPIRATORY RATE PER MINUTE
Minutes	°C.	°C.	°C.	°C.	
0	41.6	41.4	Normal	Normal	45
10	41.4	43.0	56	10	45
40	41.7	43.0	56	10	36
80	41.8	44.4	60	9	32
120	42.0	45.0	60	7	34
Cold collar removed from neck					
123	42.1	45.0	60	Off	150
140	42.6	45.4	56	Off	200

TABLE 4

Showing typical effects of increasing humidity and temperature upon panting responses of adult pigeons

Twenty experiments were conducted upon 5 birds

STARTING TEMPERATURE	AVERAGE RELATIVE HUMIDITY	PANTING THRESHOLD	MINUTES TO REACH THRESHOLD	THERMAL TOLERANCE
°C.				
42.5	20 ± 5%	43.1	37	0.6°
42.6	34 ± 5%	43.1	20	0.5
42.5	48 ± 5%	43.2	15	0.7
41.5	54 ± 5%	42.5	10	1.0
42.7	75 ± 5%	43.0	5	0.3

In young birds the lethal temperature varied more than in adults, the range of variation being between 46.0 and 47.8°. However, an average of 47.0° compares closely with the less variable average found in adults. Administration of oxygen always maintained respiratory and heart functions to somewhat higher temperatures, and the lethal temperature was in all instances higher than in those experiments without oxygen therapy. Temperatures as high as 48.8° were recorded before sudden muscle atony, arrhythmia, and final heart failure ended in death.

DISCUSSION. As pointed out by Baldwin and Kendeigh (1932), the mass or size of the body of birds increases proportionately faster than does surface area. Thus as more protoplasmic tissue is involved in heat production with comparatively little change in area for dissipation of heat, the normal body temperature must rise.

Evidence indicates that chemical mechanisms of temperature control are developed during incubation and in the first few days of the post-hatching period. It has been noted by others that the period of maximal basal heat production and growth occurs fifteen days after hatching, a period which corresponds closely with the attainment of adult body temperature levels as shown in figure 1. Further control then depends upon the formation of an external insulation of feathers.

Since the heat regulatory mechanism of young chicks is functional within a few hours after hatching, it appears that the chick is potentially homoiothermic at hatching, but upon exposure to temperature extremes, may revert toward the poikilothermic state of its embryological development.

Shivering in birds appears to consist of two separate and distinct physiological components. The first is reflex shivering initiated by impulses arising in skin receptors (possibly following an increased metabolism due to hormonal control). Reflexly increased breathing rates represent increased muscular contraction designed to raise metabolic production of heat. Secondly, since shivering may be produced while deep body temperature is decreased but skin temperature is held constant, a centrally initiated shivering is indicated. General depression of all oxidative functions is probably involved in cessation of shivering at temperatures below 20°C.

The lack of oxygen seems the significant cause of death at low temperatures. Drastic slowing of the heart results in failure to circulate sufficient oxygen to the vital centers of the brain and breathing fails. The remarkable resistance of the "warm-blooded" heart of the young chick is demonstrated in the intrinsic beat maintained at temperatures below 10.0°C. Such endurance compares favorably with poikilothermic hearts.

Water loss occurs mainly in the mouth and pharynx as air is passed rapidly back and forth over these areas. The temperature of inspired air, together with humidity, controls to a large extent the amount of heat which can be lost by panting. As the temperature rises, relative humidity of environmental air decreases, and as a result, an increased amount of evaporation from expired air is possible. If, however, the relative humidity is raised simultaneously, evapora-

tion necessarily varies in an opposite direction, i.e., less water evaporates and less heat is lost.

It is of interest to note that the lethal temperature of birds, which is two to three degrees higher than that of mammals, can be correlated with the normal body temperature which is three to five degrees higher. (*This information emphasizes the important physiological and ecological fact that homoiothermic animals are living in the upper limits of their tolerable temperature range.*)

The exceedingly rapid rate of panting in birds has been shown to be due to overheating of a panting center located in the brainstem above the respiratory centers in the medulla (von Saalfeld, 1936). When the body temperature exceeds the threshold of this center polypnea is initiated by the respiratory centers as influenced by the higher center. With continuous hyperthermia, the failure of respiration just prior to death may indicate one, or a combination of, the following events: the panting center ceases firing, the impulses are blocked en route to the medullary centers, or the latter centers are rendered insensitive to the descending impulses. Failure of all breathing movements soon after the preliminary slowing may be suggestive of the last possibility. It has recently been found in our laboratories that birds are greatly dependent upon the integrity of the vagi for functional panting, and it is thus possible that vagal failure may be involved.

Acute anoxia is certainly important in the causation of death at high temperatures and the usual sequence of events would appear to be: paralysis of the respiratory centers and consequent respiratory failure (possibly following damage to thermotaxic centers), heart and circulatory arrest due to asphyxia, and finally cessation of all oxidative functions due to tissue anoxia.

SUMMARY

1. Normal body temperature of the barred-rock chick increases from a temperature identical with that of its environment (incubator at 38 to 39°C) to about 41° ten days after hatching, after which time it approaches and remains within the limits of the dirunal variation of the adult.

2. The chick is capable of panting and shivering at or shortly after hatching, but neither mechanism is efficient in maintaining body temperature until several days of post-hatching development.

3. Development of temperature regulation is correlated with increasing metabolism and body temperature, stabilization of central thermotaxic control, and the transition from down feathers to the adult type of plumage.

4. Reflex shivering may be evoked through skin receptors and shivering may also be centrally initiated by cooling of brain centers. Reflex panting could not be demonstrated in birds; only a discrete central control of panting is believed to exist.

5. When exposed to cold environmental temperatures, periodic muscle tremors, vasoconstriction, and increased breathing and heart rates are observed. With continued hypothermia, shivering becomes continuous until, like heart and breathing rates, it progressively declines. Shivering ceases at approxi-

mately 20°C, breathing stops at about 15°C, but the heart may continue to beat at temperatures below 10°C. Respiratory and heart functions can be maintained at even lower temperatures with oxygen.

6. Neither the panting threshold nor thermal tolerance of pigeons was significantly altered at high temperatures and humidity, but severe hyperthermia developed rapidly.

7. Although some variation exists in the lethal temperature of immature birds, an average lethal temperature for fowls may be taken as 47.0°C. The sequence of physiological events leading up to high temperature death is presented and discussed.

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RESPIRATORY AND CIRCULATORY RESPONSES TO ACUTE METHEMOGLOBINEMIA PRODUCED BY ANILINE[1]

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Methemoglobinemia may be produced by a considerable number of drugs and toxic agents (1, 2). The symptoms in methemoglobinemia (3, 4) are generally similar to those caused by an equivalent degree of CO-hemoglobinemia, and this is to be expected since the mechanisms by which they produce anoxia are similar. The various methemoglobin forming agents differ in the amounts of methemoglobin they produce in various species, and also they differ qualitatively in their other pharmacological actions. Since the symptoms in methemoglobinemia have often been attributed to the direct toxic actions of the methemoglobin producing agent, the mechanism and effects of methemoglobin anoxia require further investigation in the light of current concepts of the factors controlling respiration and circulation.

In the course of investigations on a series of aromatic amino derivatives the acute actions of aniline were studied in some detail (5). It was found that in the dog the production of methemoglobinemia was the most important action and apparently accounted for most of the resulting symptoms. Small doses produced a marked and fairly prolonged methemoglobinemia without significantly altering the blood pressure. These investigations suggested that aniline could be employed as a methemoglobin producing agent for a study of the effects of methemoglobinemia on respiration and circulation.

The fact that methemoglobinemia is accompanied by a corresponding decrease in the O_2 capacity of the blood is well recognized. However, no quantitative data could be found correlating respiration during methemoglobinemia with pH and gas contents of arterial blood. Similarly no data on cardiac output appear to be available, nor data on A-V O_2 differences and estimates of venous O_2 saturation.

Therefore, a series of experiments have been carried out on unanesthetized dogs to determine the effect of methemoglobinemia on pulmonary ventilation, cardiac output, and the pH and gas contents of arterial and venous blood.

Methods. The experiments were performed on unanesthetized trained dogs after 16 to 20 hours' fasting. Each observation consisted of measuring ventilation and O_2 consumption and of obtaining samples of arterial and mixed venous blood. A control determination was made in the morning, aniline was then administered, and the experimental observation carried out $3\frac{1}{2}$ to 9 hours later. In some instances only control or experimental observations were made in a single day.

[1] A preliminary report was presented before the Society for Pharmacology and Experimental Therapeutics, Boston, 1942. Federation Proceedings **1:** 147, 1942.

Methemoglobinemia was produced by the oral administration of aniline, 50 mgm. per kgm., as a 2 per cent solution. We have shown that in dogs this dosage produces a maximal methemoglobinemia of 50 to 65 per cent in 4 hours, which persists for approximately another 4 hours and then progressively diminishes (5). At least two weeks intervened between successive administrations of aniline.

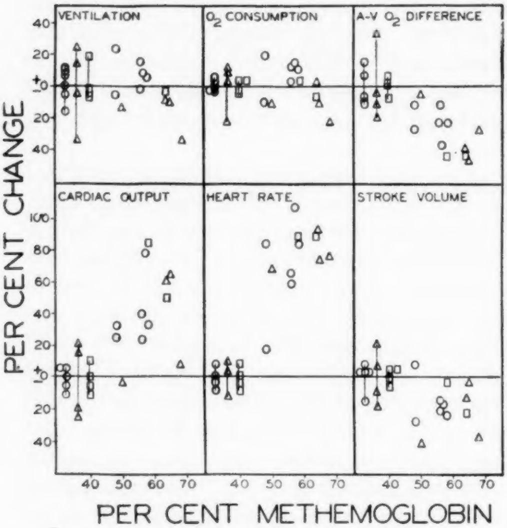
Strict conditions for basal metabolism were maintained; the preliminary rest period was never less than 30 minutes and was usually 45 to 60 minutes. A mask was fitted over the dog's muzzle and connected through rubber valves to outside air. The expired air was collected in a 60 liter spirometer and analyzed with the Haldane-Henderson-Bailey apparatus. The volume of expired air was reduced to 0°C., 760 mm. Hg, and dryness for calculating metabolism, and to 37°C., prevailing barometric pressure and complete saturation with water for pulmonary ventilation. The heart rate was counted from the femoral pulse during the 4 to 8 minutes of expired air collection which immediately preceded the blood sampling. Mixed venous blood was drawn from the right heart, and arterial from the femoral artery. Cardiac output was calculated utilizing the Fick principle (see (6) for details of the method). Rectal temperature was noted in each experiment. Room temperature during the entire series varied from 21 to 26°C. However, the change during an individual experiment was never more than 1.5°C.

Determinations were made of arterial and venous blood pH (7), CO_2 and O_2 contents (8), and cell volume (9), and of arterial O_2 capacity (after equilibration with room air at room temperature (8)), total hemoglobin (THb) and methemoglobin (MHb) (10). Corrections were made for physically dissolved O_2 (11). The blood samples were collected in oiled syringes, and mixed under oil with heparin in iced containers. Samples for gas analyses and THb and MHb were immediately transferred to mercury tonometers containing dried fluoride and analyzed within an hour. The heparinized samples were used for the pH and cell volume determinations.

The values for MHb are expressed as percentages of the THb. The percentage arterial O_2 saturation was calculated from the O_2 content and capacity using the formula $HbO_2(100)/Hb + HbO_2$. Venous O_2 saturation was calculated similarly using the venous O_2 content and arterial O_2 capacity.

Results. Twenty-six experiments were carried out on 3 dogs. There were 14 control determinations, and 12 observations following aniline administration with MHb concentrations varying from 48 to 68 per cent. The results are summarized in figures 1 and 2, and four representative experiments are shown in table 1.

The most important change in arterial blood was a reduction in O_2 capacity. The arterial O_2 saturation of the active hemoglobin remained practically unchanged since the decrease in O_2 content paralleled O_2 capacity. There were no significant changes in THb and cell volume. Arterial pH was not greatly altered. In dog 1 some tendency to a decrease was observed, but in dog 2 the tendency was toward an increase which was really significant in only one experi-



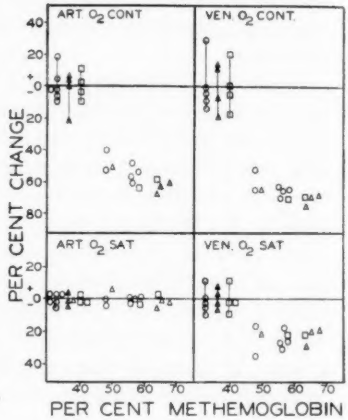


Fig. 1 Fig. 2

Fig. 1. Effects of methemoglobinemia on ventilation and circulation. The vertically connected points show the deviation from the average control value for each dog. The changes during methemoglobinemia are calculated from the control of each experiment if available, otherwise from the average control value for each dog. Dog 1 —○, 2 —△, 3 —□.

Fig. 2. Effects of methemoglobinemia on arterial and venous blood. The conventions are the same as in figure 1.

TABLE 1

DOG NO.	VENTILATION	O_2 USED	A-V O_2 DIFF.	CARDIAC OUTPUT	HEART RATE	ARTERIAL BLOOD pH	ARTERIAL BLOOD CO_2 Cont.	ARTERIAL BLOOD O_2 Cont.	ARTERIAL BLOOD O_2 Sat.	VENOUS O_2 SAT.	METHEMOGLOBIN
	l./min.	*cc./min.*	*cc.*	*l./min.*			*vol. per cent*	*vol. per cent*	*per cent*	*per cent*	*per cent*
1	4.7	84	46	1.8	55	7.31	40.0	18.0	99	74	0
	4.6	86	35	2.5	91	7.29	39.6	7.5	99	53	56
2	5.7	94	40	2.3	71	7.34	40.2	19.3	99	75	0
	3.8	73	29	2.5	125	7.37	43.4	7.5	97	60	68
	5.2	93	60	1.6	66	7.34	44.6	18.6	98	67	0
	4.7	82	32	2.6	114	7.35	44.6	7.1	97	53	65
3	5.4	93	53	1.8	56	7.33	40.4	15.5	97	64	0
	5.2	87	32	2.7	107	7.35	43.5	6.4	99	50	64

ment. Arterial total CO_2 content was variable, and in some instances the change could not be correlated with the changes in pH and ventilation.

Ventilation remained unchanged in most of these experiments; a slight decrease was observed more frequently than a slight increase. Also in 10 other experiments under similar basal conditions, no hyperpnea was noted. In some of these latter experiments the effect of inhalation of pure O_2 for 5 minutes was tested. Breathing pure O_2 produced no significant decrease in ventilation as compared to the controls.

The O_2 consumption did not vary more than $\pm$ 15 per cent although in 2 individual experiments it was slightly greater. Rectal temperature remained unchanged, or decreased slightly. Only in dog 2 was a decreased rectal temperature correlated with a decreased O_2 consumption and ventilation.

Venous O_2 content was markedly reduced. This decrease did not exactly parallel the decrease in O_2 capacity (or the increase in MHb) because in most instances there was a decrease in A-V O_2 difference. Venous O_2 saturation was significantly decreased in most cases.

The A-V O_2 difference was usually decreased without a great change in O_2 consumption. Consequently in all experiments except two an increase in cardiac output was observed. The acceleration in pulse rate was frequently greater than the increase in cardiac output and in those cases the stroke volume was decreased.

Discussion. The foregoing results were obtained on trained unanesthetized dogs and are therefore free from the effects of anesthesia. We wish to emphasize that these are relatively acute experiments with a maximum methemoglobinemia of 48 to 68 per cent produced by aniline. The mechanism of the MHb formation has been recently reviewed (5, 12), and experimental evidence of the respiratory and circulatory effects of aniline has been presented (5). From this evidence (5) we are convinced that the effects reported in this paper resulting from a small oral dose of aniline are due predominantly to MHb anoxia. At present we are not aware of any agent entirely free from other pharmacological actions which could have been employed to produce the methemoglobinemia.

Respiration does not appear to be stimulated by methemoglobinemia *per se*. It is now well established that respiratory stimulation in anoxemia is dependent upon stimulation of the carotid and aortic chemoreceptors (13). The effective stimulus for these receptors is a diminished arterial O_2 tension. A decreased arterial O_2 content without a sufficient decrease in pO_2 is ineffective as shown by Comroe and Schmidt (13b) by perfusing the carotid body with blood containing HbCO at normal pO_2. Anoxia has only a depressant effect on the respiratory center itself (13c, d). In our experiments we have not observed a significant decrease in arterial O_2 saturation and there is no reason to believe that arterial pO_2 is markedly altered. Therefore, no respiratory stimulation would be expected. On the other hand, some respiratory depression might have been anticipated. Our experiments produce no conclusive evidence on the latter point.

Definite circulatory stimulation was evidenced by the increased cardiac output. This was associated with a marked acceleration in pulse rate and some tendency to a reduction of stroke volume. Although blood pressures are not reported in this paper, observations on other animals under similar conditions have shown no great change.

The increased cardiac output clearly represents a compensatory response of the circulatory system. It is evident that a decreased arterial O_2 saturation cannot be evoked as an explanation. Some diminution of tissue pO_2 undoubtedly occurred since the venous O_2 saturation falls despite the increased cardiac output. The mechanism by which tissue anoxia (in the absence of a decreased arterial O_2 saturation) causes an increased cardiac output is obscure. Nevertheless, an increased cardiac output has been reported in acute carbon monoxide poisoning of dogs and man (14), and in anemia of man (15, 16) and of the dog (17). The rôles played by the increased pulse rate and vasomotor reactions in causing an increased cardiac output in this type of anoxia remain to be investigated.

MHb anoxia is due to a reduction in the O_2 carrying capacity of the blood. This results in a decrease in venous O_2 saturation, and consequently in venous pO_2. Darling and Roughton (12) have reported that MHb causes a definite shift to the left of the normal O_2 dissocation curve of hemoglobin. This shift will therefore cause a considerably greater decrease in venous pO_2 than is indicated by the venous O_2 saturation. For example, in an experiment on dog 2 with 68 per cent MHb, the venous O_2 saturation is 60 per cent. Approximation from the normal O_2 dissociation curve (18) gives a pO_2 of 33 mm. Hg, but from the MHb O_2 dissociation curve (12) only 19 mm. Hg. We have not shown estimates of pO_2 in this paper since the data of Darling and Roughton on dog blood are insufficient to permit this.

We have previously reported that MHb concentrations of 70 to 75 per cent in dogs caused unconsciousness, and motor release phenomena indicating cerebral depression (5). In the experiments here reported no gross neurological signs were evident other than slight lethargy, except in dog 3 which showed some neuromuscular weakness.

Our results on MHb anoxia are qualitatively similar to those reported for carbon monoxide poisoning by Chiodi, Dill, Consolazio and Horvath (14). In CO-hemoglobin anoxia the circulatory effects were apparent at somewhat lower concentrations of inactive hemoglobin, as might be expected from the more marked effect of HbCO on the O_2 dissociation curve. Since carbon monoxide is free from the objections which may be made against agents producing MHb, these observations would appear to lend support to our interpretation of the effects of methemoglobinemia.

SUMMARY

1. The effects of MHb concentrations of 48 to 68 per cent, produced by an oral dose of 50 mgm. per kgm. of aniline on trained unanesthetized dogs are described.
2. No respiratory stimulation was observed.
3. Cardiac output and pulse rate were significantly increased.
4. Arterial O_2 content was reduced in direct proportion to the MHb, but arterial O_2 saturation remained unchanged.
5. Venous O_2 saturation decreased despite the increased cardiac output.

6. The effects of methemoglobinemia on arterial and venous O_2 tension are discussed.

Acknowledgment. The authors wish to express their appreciation to Dr. Cornelius Daly, formerly of this laboratory, for designing and setting up the pH apparatus and for valuable assistance in planning these experiments.

We also wish to thank John Romano for technical assistance.

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DIETARY "SELF-SELECTION" AND APPETITES OF UNTREATED AND TREATED ADRENALECTOMIZED RATS[1]

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Richter (1–4) has described certain beneficial changes in appetite and food, water, and electrolyte selection in self-selection dietary experiments with adrenalectomized rats. He has observed decreased sodium taste thresholds with increased sodium intake (5–9), certain changes in water intake (3, 5, 6, 8, 9), and lastly, a decreased carbohydrate intake (6, 7), when adrenalectomized rats were allowed to select sodium solutions and sugar from separate feeders and drinkers. These results and those obtained in other studies by Richter, have been used to support certain claims purporting to partially elucidate "biological drives," "behavior," "adaptability," "effort to maintain a constant internal environment," and so forth.

It is obvious that if Richter's claims could be confirmed, such phenomena would be of fundamental biological importance. When Richter's work (6, 7) appeared on sugar and salt appetites of self-selecting adrenalectomized rats, we had been working along somewhat similar lines, and since we used a somewhat different technique and obtained different results, it was thought of interest to present these findings at the present time.

METHODS. Rats of uniform weight and sex, of a strain from which the Wistar colony originated (10), were divided into two groups of three to ten rats each, and placed into two cages, in which the various components of an adequate synthetic diet were separately offered in different feeders and drinkers. The cages were made of one-half inch hardware screen and were of the approximate dimensions, 18 inches x 13 inches x 8 inches deep. A refuse drawer was built under each cage, and clamp holders for the various drinkers were soldered to the backs of the cages. The drinkers were constructed from inverted glass graduated cylinders, with attached glass spouts, the spouts protruding into the cages, and enclosed within individual horizontal metal cylinders of approximate diameters of 2 inches, to prevent the rats from walking on the spout openings and thus causing loss of fluid from the openings on the upper faces of the spouts.

Cylindrical metal feeders 3 inches x 3 inches were used, which had removable telescoping tops with a 1.5 inch hole. The outside bottoms were leaded to prevent overturning of the feeders. Food was not spilled because of the restricted entrance.

The rats were separately offered in these feeders: yeast (Fleischmann's dried

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brewer's yeast, Type 2019), dextrose (Corn Products "Ceralose"), casein (Labco vitamin-free, 100 mesh), and salt mixture (SMACO, USP XI). Roughage was offered as filter paper scraps, in a wire mesh feeder attached to the cage. The rats were offered the following fluids, from inverted 250 ml. or 100 ml. graduated cylinders, with spout drinkers: 3 per cent NaCl (USP) 250 ml., distilled water 250 ml., and a mixture of corn oil (Corn Products "Mazola") and codliver oil (USP) in the proportion of 4 to 1, 100 ml.

Preliminary experiments with some twenty rats were performed, in which various salt solutions were offered in different 100 ml. drinkers, including: water, 3 per cent NaCl, 1 per cent KCl, 2.5 per cent $MgCl_2$, 4 per cent NaH_2PO_4, 2.4 per cent Ca-lactate (or pyruvate or levulinate), corn oil, and codliver oil. These experiments were designed to reproduce some of the conditions imposed by Richter's earlier experiments on salt appetite after adrenalectomy (8). The solid salt mixture was not offered in these experiments. The rats selected successfully in a few cases, as judged by comparison of their growth curves with those of comparable rats fed Purina fox chow or an adequate mixed diet, but subsequent experiments showed that more consistent and reproducible results were obtained when the solid salt mixture was offered in place of the multiple choice of solutions. We therefore later offered only water and 3 per cent NaCl solutions, since essential minerals were available in the complete salt mixture and in the yeast.

Prior to self-selection experiments, the animals were fed on our stock diet; Purina fox chow supplemented once weekly with greens.

The food and fluid intakes, and the body weights were measured at the same time of day every other day for long periods of time, in order to establish the time at which relatively constant and comparable food and fluid intakes were established. After this time, one group of animals was bilaterally adrenalectomized, and the other was either sham-operated or unilaterally adrenalectomized.

The procedure of adrenalectomy has been described elsewhere (11), with certain modifications. Employing ether anesthesia and using the dorsal lumber approach without sterile precautions, this procedure is very similar to that recently described by Richter (7). Such bilaterally adrenalectomized rats, of the particular strain used, survived on the average of four to seven days for 100 gram rats, and thirteen to thirty days for mature rats, when they were fed a low sodium diet made of vitamin-free casein, dextrose, yeast, codliver oil, corn oil, and a sodium-free modification of the salt mixture described by Hubbell et al. (12), in which potassium salts were substituted for the sodium salts described by these authors. The animals used in the experiments to be described generally all succumbed at the conclusion of the experiment, when sodium chloride or adrenal cortical hormone therapy was withheld. The rare exceptions which did not succumb at this time were carefully autopsied and examined for accessory adrenal cortical tissue or rests, in order to see if the experiment was thereby invalidated.

In some experiments which will be described, diets were mixed in proportions dictated by changes in intake seen in self-selection experiments with adrenalec-

tomized rats, and fed to a uniformly selected group of eight to ten rats, as compared with another group fed a diet composed of normal ratios of dietary constituents, such as: dextrose 58 per cent, casein 21, corn oil 7, codliver oil 2, yeast 10, salt mixture 2, and containing 4.2 calories per gram. These experiments were designed to see if diets made according to the diets selected by adrenalectomized rats, would benefit adrenalectomized rats or alter their voluntary intake of sodium chloride.

In all, six successful self-selection experiments were performed, involving some eighty rats, over a two-year period. Each experiment involved two groups of rats, one control and one experimental group. The rats of each group were housed together in one cage, and consisted of from three to ten rats each, depending upon the type of experiment. The experiments lasted an average of three to four months each. In this way food and fluid intakes represented the average of all the rats of one group rather than individual rats.

In general, adrenalectomy was not performed until the rats had established constant food and fluid intake levels. This period varied from twenty to thirty days in most cases. Intakes and body weights were nevertheless recorded for this period every two days. Richter (7) usually found constant levels established by the fortieth day.

Of the experiments performed, only one will be described in detail, since the experiments were all essentially similar, and revealed certain consistencies exemplified by this one.

Experimental. *Experiment 4.* Three month old female rats averaging 156 grams body weight (range 140 to 170) were divided into two groups of ten rats each, and each group was separately caged, and offered casein, dextrose, yeast, salt mixture (dry), water, corn-codliver oil mixture, and three per cent NaCl, as described in Methods.

The body weights and food fluid intakes were recorded every two days for a period of ninety days. The solutions in the drinkers, and the food in the various feeders were changed for fresh supplies every two days. The amount of evaporation fro: . the spouts of the drinkers in the two day period, was neglected.

The rats of group 1, the controls, were unilaterally adrenalectomized, and those of group 2, the experimentals, were bilaterally adrenalectomized on the forty-seventh day. The operation was performed at this late time because prior to this time the water intake was not equal in the two groups, as seen in figure 1. Twenty days after the operation, a fifty-milligram pellet of desoxycorticosterone acetate[2] was subcutaneously implanted in the shoulder region of the rats of group 2, and removed twenty-five days later, and the rats subsequently given the low sodium mixed diet previously described, in addition to 0.1 per cent KCl drinking fluid, which was increased to 0.25 per cent a week later. The food intakes were averaged as per cent calories per rat per day, and the water and NaCl solutions were recorded as milliliters per rat per day. The caloric equivalents per gram of the diets selected by the rats were assumed to be: dextrose 3.75, casein 4.40, oils 9.30 (13) and yeast 2.0 (assumed 50 per cent protein, and

[2] Supplied by Roche-Organon, Inc., through the courtesy of Dr. R. D. Shaner.

negligible in fats and carbohydrate, according to analyses obtained from Fleischmann's).

The results were averaged for five-day intervals for convenience in graphing, and the results are presented in figure 1.

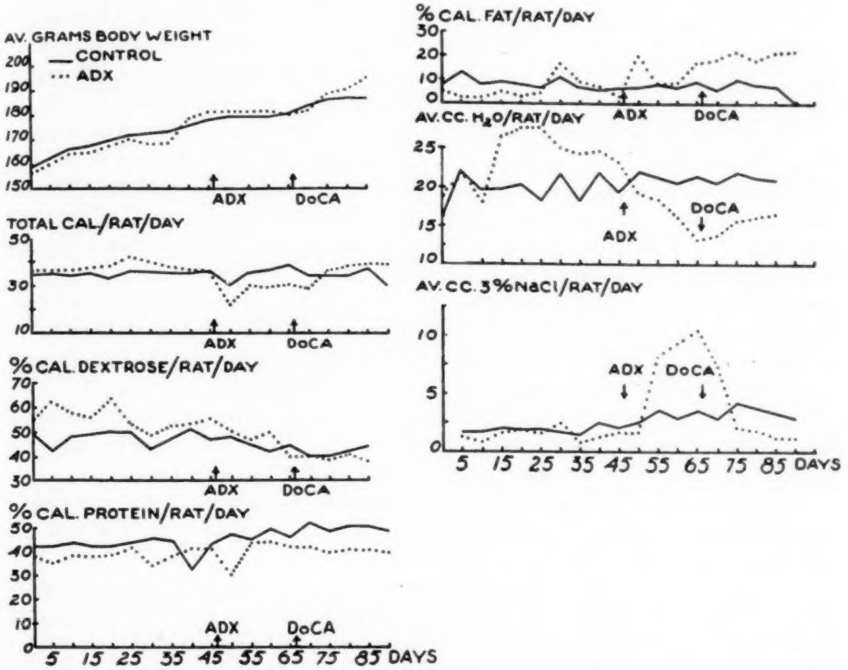

Fig. 1. Effect of adrenalectomy on voluntary selection of dietary constituents separately offered.

Constituents separately offered were dry dextrose, yeast, casein, and salt mixture; corn oil-codliver oil (4:1), 3 per cent NaCl, and water. Ten rats per group. Each group caged together. Each graph average of the 10 rats.

ADX—adrenalectomized. DoCA—Desoxycorticosterone acetate.

As can be seen from figure 1, the body weight curves showed no effect of the operations. After removal of the desoxycorticosterone acetate pellets, the animals died in 100 per cent of the cases when they had no access to salt, after an average of twenty-six days (range 9 to 36). The average body weights are not plotted after pellet removal since the scattered times invalidated the significance of such averages.

The total caloric intake showed a small transitory decrease caused by the bilateral adrenalectomy in group 2, which slowly returned to normal, especially after desoxycorticosterone acetate administration. Otherwise no significant difference was seen between the two groups.

The percent calories of dextrose showed no appreciable difference between the two groups after adrenalectomy, and the same lack of effect was seen in the protein (including yeast) and fat intakes, although transitory variations in fat intake occurred. Since the caloric equivalent of fat is high, the actual intake in milliliters was exaggerated by multiplication by this factor, 9.3, and the variations were much less noticeable if milliliters per rat per day were recorded instead of calories per cent per day. There was no significant change in the intake of the dry salt mixture, which was consumed on the average of about 100 mgm. per rat per day, but which showed considerable variation from day to day.

The water intake curves were not comparable between the two groups prior to a few days before the operations. After operation, the water intake of the bilaterally adrenalectomized rats decreased from 20 to approximately 13 ml. per rat per day, while the 3 per cent NaCl intake increased from 2 ml. per rat per day, to a maximum of 10 ml. per rat per day, a fivefold increase due to adrenalectomy, in confirmation of Richter. After desoxycorticosterone acetate administration the salt intake again returned to the pre-adrenalectomy level, while the water tended again to increase. Due to the difference in water intakes of the two groups prior to adrenalectomy, it might be believed that the changes in water intake after operation have no significance in this experiment. This may be true, but since the same tendency to decrease water intake and increase 3 per cent NaCl after adrenalectomy, was seen in the other experiments, we believe that the water decrease tends to parallel the salt increase.

The results show that we could confirm Richter's observations that adrenalectomy increases voluntary intake of sodium chloride, but we could not confirm his observation that selection of sugar decreases.

The results of the other experiments were similar to this one, with some exceptions. These exceptions were that upon occasion, the two groups of rats would sometimes not establish comparable intake levels of fluids and foods prior to adrenalectomy, as in the case of water in experiment 4, described above. In all, however, the experiments showed essentially the same results, namely, that adrenalectomy increases the voluntary intake of sodium chloride, but does not significantly alter the voluntary intake of any of the other dietary constituents, with the possible exception of water.

An outstanding exception was experiment 2, in which there were seven rats per group and the methods used were essentially the same as those described in experiment 4. In this experiment, the total caloric intake remained about the same after adrenalectomy and after desoxycorticosterone acetate or adrenal cortical extract administration. As in experiment 4, the NaCl intake increased after adrenalectomy, and decreased after desoxycorticosterone acetate, but the water intake remained the same. The fat intake, however, markedly increased and the dextrose intake markedly decreased after adrenalectomy in such a way as to render the total caloric intake unchanged. The decreased dextrose intake is in agreement with Richter's experiments, but in disagreement with our other experiments. In this particular experiment, however, after adrenalectomy, even though the rats had access to and voluntarily increased their NaCl intake,

it was noticed that their body weights fell lower than the controls, and several rats died during the time with severe diarrhea. All the rats of this group were markedly asthenic. This type of result has been described by Mark (14), whose work is described in the discussion below. In no other experiment, in which all the rats were healthy and vigorous, could a change in food intake be observed for any of the dietary constituents except NaCl (and possibly water).

On the occasions in which experiments showed changes in voluntary intake of the dietary constituents due to unknown causes or to poor condition as in experiment 2, it was thought of possible interest to mix synthetic diets, with their proportions of constituents dictated by these self-selection experiments. The first such experiment consisted of six groups of five female rats per group, average body weight 160 grams. Three groups, the controls, were fed a diet dictated by normal rats in a previous self-selection experiment, and three groups, the experimentals, on a diet dictated by adrenalectomized rats in the same experiment, in which the rats selected a high protein, low carbohydrate diet. In this experiment, as in experiment 2, the rats did poorly, and did not reversibly alter their voluntary NaCl intake after adrenalectomy and subsequent hormone therapy. The three control groups were fed a mixed diet consisting of: dextrose 58 grams per cent, casein 21, corn oil 7, codliver oil 2, yeast 10, and salt mixture 2, and contained 4.16 calories per gram, and the calories per cent of the various constituents were: carbohydrate 52, protein 28, and fats 20. The experimental groups were fed a diet consisting of: dextrose 28 grams per cent, casein 40, corn oil 10, yeast 10, codliver oil 2, salt mixture 2, and celluflour 8; containing 4.15 calories per gram, and consisting of: carbohydrate 25 calories per cent, protein 48, and fats 27.

The three control groups and the three experimental groups were given, respectively, tap water, 0.5 per cent NaCl, and 1.0 per cent NaCl as drinking fluid, and the body weights, food and fluid intakes were recorded for a week, after which the experimental groups were adrenalectomized, and the data recorded until death. Survival times were noted.

No differences were observed for food and fluid intakes, or for body weight changes after adrenalectomy, except that the rats on 0.5 and 1.0 per cent NaCl as drinking fluid ate and gained more weight than those on tap water, which is to be expected. After removal of the NaCl, no differences were noted in survival times between the control and experimental groups. This experiment indicates that the high protein, low carbohydrate diet dictated by the previously conducted self-selection experiment mentioned, had no beneficial effects on the rats with respect to appetite, weight maintenance, or intake ("requirement"?) of NaCl, as compared with a control diet.

In another similar experiment, four groups of eight female rats each, averaging 125 grams (range 117–138) were selected. The first group received a mixed diet dictated from the results of self-selection experiment 2, described above, in which dextrose intake decreased, and fat intake increased after adrenalectomy (but in which this change seemed to parallel a poor condition of the animals). The control groups received a mixed diet, diet 1, consisting of dextrose 62 grams

per cent, casein 20, yeast 12, corn oil 3.2, salt mixture 2.8, and containing 3.7 calories per gram, with the calories per cent of carbohydrate 62, protein 30, and fat 8; while the experimental group received a high fat, low carbohydrate diet, diet 2, consisting of dextrose 25 grams per cent, casein 20, corn oil 17.2, yeast 12, salt mixture 2.8, celluflour 23, and containing 3.7 calories per gram, with the calories per cent of carbohydrate 31, protein 26, and fat 43. Each rat received 2 drops of Oleum percomorphum (Abbott) once weekly as a source of vitamins A and D.

Both groups, in addition to receiving the diets described, were allowed a voluntary selection of two drinking fluids, tap water and 3 per cent NaCl, from two drinkers. For roughage, both groups had access to filter paper scraps. It was noted, however, that the rats of the experimental group did not consume the paper while those of the control group did. This was presumably because of the lack of roughage in the control diet (self-selection?). One control and one experimental group were then adrenalectomized, leaving the other two as non-adrenalectomized controls. The adrenalectomized animals were operated three weeks prior to the experiment, and maintained on salt therapy consisting of a 1 per cent NaCl, 0.2 per cent $NaHCO_3$ drinking fluid. Just prior to the presentation of the experimental diets, these animals were tested for completeness of adrenalectomy by withholding salt therapy, and all the rats showed a marked weight loss. After this, they were given 2 mgm. desoxycorticosterone acetate in oil subcutaneously, which restored them to normal weight and health. Thus the rats were proven, prior to the experiment, to be capable of developing a severe uniform adrenal insufficiency.

Body weights, food and fluid intake, and voluntary intake of salt or water were recorded daily. After 32 days, the 3 per cent NaCl solution was removed, and the weight loss and survival noted.

The "dictated" diets had no effect on food intake, water intake, or salt appetite for the 32 day experiment, nor were there any differences in survival after withdrawing salt, the survival time being 6 to 7 days, respectively, for the two groups of adrenalectomized rats on the two diets. It is noted that the adrenalectomized rats did select much more NaCl than the controls, in confirmation of the self-selection experiments, and those of Richter. The controls selected an average of 200 mgm. NaCl per rat per day, while the adrenalectomized rats selected an average of 400 mgm. per rat per day.

Another similar experiment was performed with mature female rats, in contrast to the young rats used in the experiment just described, with essentially the same results.

Discussion. In the self-selection experiments described above, all of the dietary constituents were separately offered in different feeders and drinkers, and the salts were offered as dry salt mixture, with exception of a NaCl solution. These experiments differ from those of Richter, in which voluntary dextrose and salt intakes were observed to be altered by adrenal insufficiency. In one series of experiments, Richter (7) fed a saltless stock diet in one container, and 3 per cent NaCl, 40 per cent dextrose, and distilled water from three drink-

ers, and found that adrenalectomy decreased the intake of dextrose solution, increased that of the salt solution, and decreased the water intake. Desoxycorticosterone acetate and adrenal cortical extract tended to reverse this change. In another series of experiments (6), he fed the same diet from one container, 3 per cent NaCl and water from two drinkers, and dry dextrose from another feeder, with essentially the same results.

In the experiments we performed, in which diets were made up according to the proportions of various dietary constituents selected in certain self-selection experiments in which changes in intake were observed after adrenalectomy (it is pointed out that these experiments were exceptions), no effects of altered fat, carbohydrate, or protein content of the diets were observed on appetite, body weight, salt selection, or survival of adrenalectomized rats fed such diets. This is in agreement with the experiments of Swann (15) who examined the effects of diet on the survival of 704 immature and mature adrenalectomized rats of four different strains, and was unable to find any influence on survival, of variations of carbohydrate, fat or protein content.

In addition to the experiments we have performed, and those of Richter, Warkinton (16) has described self-selection experiments in which the rats "successfully" selected an adequate diet when presented with the separate constituents of the diet. He was interested in ascertaining whether hypo- or hyperthyroidism would alter the voluntary selection of any of the dietary constituents, but obtained negative results.

Mark (14) fed rats a saltless stock diet, and 40 per cent sugar, 3 per cent NaCl, and distilled water from three drinkers, thus essentially the same technique described by Richter (7). After adrenalectomy, the NaCl intake markedly increased, returning to normal after desoxycorticosterone acetate. No mention was made of alterations in sugar and water intakes except a statement to the effect that changes in voluntary sucrose and water intakes did not show a significant pattern. Even with access to, and increased voluntary intake of the salt solution, Mark's animals frequently died 15 to 20 days following adrenalectomy, which does not confirm Richter's 100 per cent survivals under identical conditions. Mark mentions the possibility of colony differences, but his rats' failure to maintain themselves by salt selection reminds us of our experiment 2, in which, despite an increased salt intake, several rats did poorly, and even died. It is noteworthy that experiment 2 was an exception to our general observation that adrenalectomy does not alter the voluntary intake of any of the dietary constituents when they were separately offered, in confirmation of Mark. Richter (7) mentions the possibility that the decreased sugar intake observed after adrenalectomy, when sugar is offered as the solid or as a solution in addition to a saltless stock diet, is due to impaired intestinal absorption. It is a well known fact that adrenalectomized animals cannot tolerate large amounts of hypertonic solutions such as strong sugar solutions (17), and adrenalectomized rats might therefore learn to avoid such osmotic dangers when they already have access to an adequate mixed diet in addition to an extra supply of carbohydrate in the form of solid dextrose or strong solutions of dextrose. When the

dietary constituents are separated in different feeders, however, we feel that adrenalectomy does not alter the voluntary intake ("need") of carbohydrate, or any other dietary constituent except salt and water. Richter's observation of a decreased sugar intake might be explained on the basis of an impaired sodium balance (18).

SUMMARY

1. Self-selection experiments were performed on rats, in which the effects of adrenalectomy were studied, in an attempt to see if the voluntary intake of any of the dietary constituents was altered. Dextrose, salt mixture, yeast, and casein were offered in four feeders, and 3 per cent NaCl, water, and a mixture of corn oil and codliver oil from three drinkers. Several rats were caged together. The daily intakes were recorded until constant, after which adrenalectomy was performed, and the subsequent intakes recorded for a period of time, followed by adrenal cortical hormone therapy.

2. Richter's observation of an increased voluntary selection and beneficial effect of NaCl, reversed by adrenal cortical hormone therapy, was confirmed.

3. In those experiments which were considered valid, no effect of adrenalectomy was observed on the intake of protein, carbohydrate, fat, yeast, or solid salt mixture. This differs from Richter's observation of a decreased sugar intake. It is pointed out, however, that Richter's method differed in that he presented sugar in addition to a mixed diet.

4. An exceptional experiment is described which does show a decreased sugar intake after adrenalectomy, in confirmation of Richter, but it is pointed out that the rats lost weight, and several died.

5. Diets were made up in accordance with the results of self-selection experiments, such as the exception described in paragraph 4, which were considered to show changes in self-selection of dietary constituents presumably due to the poor condition of the animals after adrenalectomy. These diets varied in carbohydrate, fat and protein content, and were fed to adrenalectomized rats. Three per cent NaCl and water were also offered. No effects of these diets, "dictated" from self-selection experiments, were observed on appetite, body weight, voluntary intake of salt solution, or survival after withholding the salt solution, even though adrenalectomy did lead to an increased salt intake.

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THE INFLUENCE OF SERUM CHLORIDE CONCENTRATION ON THE OXYGEN CONSUMPTION OF DOGS

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In the course of work involving observations on the oxygen consumption of dogs before and after the intravenous administration of various metabolic substrates, it was found that similar injections of NaCl solutions also increased the oxygen uptake. Since physiological saline is ordinarily considered to be metabolically inert and is commonly used as a vehicle for the injection of other materials, it became of practical as well as of theoretical importance to investigate its stimulating effect upon oxygen utilization.

Previous recognition of the above influence of NaCl has been largely associated with the disturbance encountered in adrenal insufficiency. It is well known that untreated adrenalectomized animals have an abnormally low oxygen consumption but that when such animals are maintained on an adequate intake of NaCl, their rate of oxygen consumption approaches that of normal animals (1) (2) (3) (4). Similarly, it has been demonstrated (in vitro) that the Q_{O_2} of isolated tissue from rats in adrenal insufficiency is lower than that of tissue removed from adrenalectomized rats treated with NaCl (5) (6). The previous literature contains only a few scattered references to the effect of NaCl on oxygen consumption in the normal organism. Castex and Schteingart (7) reported that subcutaneous injections of NaCl in human subjects raised the basal metabolic rate from 4 to 19 per cent. Control observations using distilled water gave no increase. Davis (8) (9) noted increased oxygen consumption in dogs following intravenous NaCl administration and observed that when the animals were initially dehydrated, the effect of NaCl administration was greater.

Methods. The work, to which the present observations were incidental, necessitated the employment of several different physiological states in dogs and gave us the opportunity to test the effect of NaCl under the most diverse conditions. Both normal and completely depancreatized dogs were used, sometimes intact and sometimes after complete abdominal evisceration. All animals were deeply anesthetized with nembutal throughout the measurement of the respiratory exchange. The latter procedure was carried out with an apparatus to be described in a forthcoming report on other work. All animals were deprived of food for 3 days before the experimental day, depancreatized dogs being deprived of insulin as well as food for the same period. Depletion of NaCl or of chloride alone was effected by the methods of Darrow and Yannet (10) (11). The depletion was carried out on the experimental day or on the preceding day. Sodium chloride was restored to the animals by the intravenous injections of

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isotonic or hypertonic NaCl. When it was desired to replace only the sodium ion, sodium phosphate was administered. Chloride, dissociated from sodium, was given as lithium chloride.

All analyses were made on arterial blood. Plasma sodium was determined by the method of Weinback (12) and serum chloride by Wilson and Ball's method (13). The whole blood lactic acid was measured by the method of Barker and Summerson (14).

RESULTS. Figure 1 is a graph of a typical experiment on an intact normal dog. There is a striking parallelism between the depletion and restoration of NaCl and the fall and rise in the rate of oxygen consumption. It should be noted that the administration to a normal undepleted dog of the amount of NaCl which was required to restore the depleted dog to normal, also resulted in a significant rise in the oxygen consumption (about 20 per cent). However, in the normal

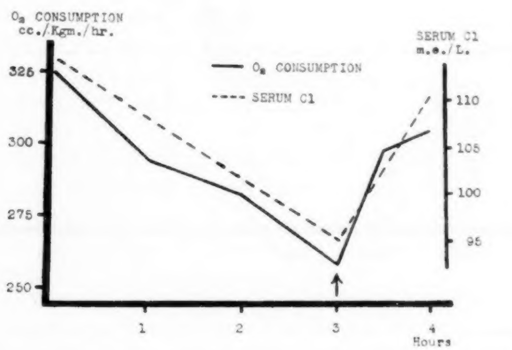

Fig. 1. A typical experiment on an intact normal dog weighing 10 kgm. It received an intraperitoneal injection of 1000 cc. of 5 per cent glucose, at zero time. The arrow represents the time of withdrawal of the fluid from the peritoneal cavity, followed by the intravenous administration of 4.7 grams of NaCl dissolved in 30 cc. of distilled water.

dog, the increase in oxygen consumption reached its peak in about 30 minutes, was maintained for another 30 minutes, and then fell to the pre-injection level about 30 minutes thereafter. In contrast to this, the administration of NaCl to a depleted dog or to an animal which was salt deficient as a result of uncontrolled pancreatic diabetes, produced an equally rapid rise in the rate of oxygen consumption, but the increased rate then persisted for as long as we continued our observations (for as long as 5 hrs.).

We have some data which indicate that the influence of NaCl on the rate of oxygen consumption is largely due to the chloride ion. By the intraperitoneal injection of a mixture of 5 per cent glucose and 150 m.e./liter $NaHCO_3$, four intact normal dogs were depleted of chloride alone. In these animals, in which the plasma sodium levels did not change, final serum chloride levels of 79, 80, 81, 83 m.e./liter were accompanied by decreases in oxygen consumption amounting to *15, 13, 43, 16* per cent respectively. Concordant results were also obtained

by depleting animals of NaCl, then administering first the sodium ion, and then the chloride ion. In three intact normal dogs, depleted to levels of *102*, *117*, *125* m.e./liter of plasma Na and *86*, *82*, *93* of serum Cl, restoration of the Na resulted in rises in oxygen consumption amounting to *10*, *6*, *9* per cent respectively. Subsequent restoration of the chloride increased the oxygen consumption *20*, *15*, *30* per cent.

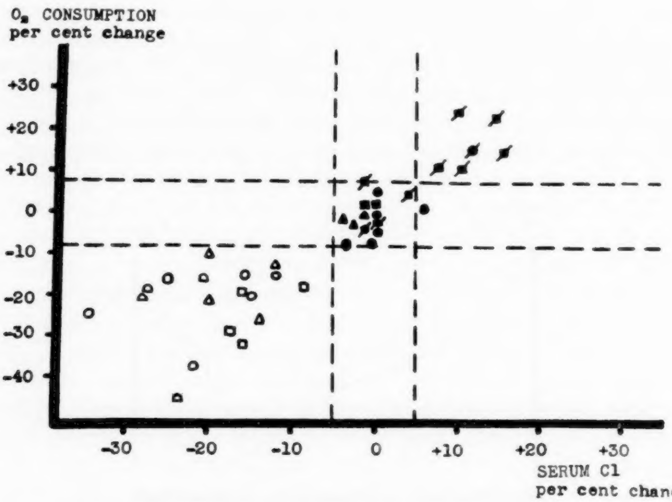

Fig. 2. A summary of all results on different animal preparations. Because of the widely different initial levels of serum chloride and oxygen consumption, the correlation shown is that between the percentage deviations from the initial levels. The deviations in chloride concentration are the result of either the abnormal state (as in the uncontrolled diabetic animals) or of the withdrawal or administration of NaCl or Cl.

Effect of NaCl depletion on normal intact dogs = ○, on eviscerated normal dogs = □, on diabetic dogs = △.

Effect of Cl depletion on normal intact dogs = ⌓.

NaCl restored to normal intact dogs = ●, to eviscerated normal dogs = ■, to diabetic dogs = ▲.

Additional NaCl given to normal intact dogs = ●́, to eviscerated normal dogs = ■́.

The broken lines indicate the approximate limits of normal variation.

Figure 2 summarizes all our results indicating the variation of oxygen consumption with variations in the concentration of chloride in the blood serum. Because the graph includes data from animals in a variety of physiological states, involving widely different initial levels of oxygen consumption and chloride concentration, the correlation presented is that between percentage deviations rather than absolute values. The direct relationship between serum chloride concentration and oxygen consumption in all the animals is apparent.

There was little indication from this work as to a possible explanation for the influence of serum chloride concentration on oxygen consumption. An incidental

observation which may be pertinent was the sharp rise in the levels of lactic acid in the blood, in a few sodium chloride depleted animals in which this was followed. The increases varied from 31 to 106 mgm. per cent. This suggested the possibility of the occurrence of a relative anoxia in the tissues of the depleted animals which might point to a defect in the oxygen-carrying or transfer mechanisms.

SUMMARY AND CONCLUSIONS

1. The influence on the total oxygen consumption of dogs of the administration of NaCl and of NaCl depletion was observed under a variety of conditions, including normal and depancreatized animals, both intact and after complete abdominal evisceration.

2. Under all these conditions, the oxygen consumption of the animals, if normal to begin with, was temporarily increased by the administration of NaCl. The total oxygen consumption of animals depleted of NaCl was abnormally low, and was permanently restored to the normal value by the administration of sufficient NaCl to bring the blood level back to normal.

3. Some of the data indicate that the influence of NaCl is largely due to its Cl ion.

4. There is a suggestion that the chloride ion is involved in the mechanisms for the transport and transfer of oxygen from blood to tissue.

5. From a practical standpoint, it is important to consider serum chloride concentration as a possible variable factor in all investigations involving measurement of the oxygen consumption of animals.

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THE INFLUENCE OF SODIUM CHLORIDE CONCENTRATION ON THE IN VITRO OXYGEN CONSUMPTION OF RAT DIAPHRAGM, IN THE PRESENCE AND ABSENCE OF RED BLOOD CELLS

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Previous authors have reported inconsistent and contradictory effects on the in vitro oxygen consumption of tissues as a result of varying the NaCl concentration of the medium (1) (2) (3) (4). Our own previous observations on the in vivo oxygen consumption of dogs have shown a consistently direct relationship between the concentration of the serum chloride and the basal oxygen consumption (5). It therefore seemed worthwhile to reinvestigate the subject under in vitro conditions, in an attempt to discover the reasons for the lack of correspondence of such results with those we obtained in the living organism.

Methods. The oxygen consumption of portions of the diaphragms of 60 to 90 gram rats was measured by the Warburg technique. A calcium-free Ringer-phosphate buffer, pH 7.4, containing 200 mgm. per cent glucose was used as the medium. The basic solution to which glucose and NaCl were added as required, contained the following concentrations of ions, expressed as m.e./liter: K = 4.4, Mg = 1.2, Na = 30, Cl = 3.0, SO_4 = 1.2, PO_4 = 20. Both glucose and NaCl were added in dry, solid state to aliquots of the basic solution to yield the following 3 final solutions: Solution no. 1 in its final form contained 150 m.e./liter of Na and 133 m.e./liter of Cl; solution no. 2 contained 200 m.e./liter of Na and 183 m.e./liter of Cl; solution no. 3 contained 100 m.e./liter of Na, 83 of Cl, and (to avoid hypotonicity) 75 m.e./liter of sucrose. For convenience, these solutions are hereinafter designated as NaCl at 150, 200 and 100 m.e./liter respectively.

Every experiment was performed in duplicate for an observation period of one hour and consisted of a comparison of control vessels containing the physiologically normal NaCl concentration of 150 m.e./liter (solution no. 1) with test vessels containing solutions no. 2 and 3. In each experiment, portions from the same diaphragms were compared in the different solutions. Q_{O_2}'s were determined on the basis of dry weight of the tissues.

Two other variables tested in these experiments were the gas phase (100 per cent oxygen compared with air) and the presence or absence of red blood cells in the medium. When red blood cells were used, they were obtained from oxalated, defibrinated, heparinized or citrated dog or human blood. They were washed 5 or 6 times with 0.9 per cent NaCl solution, oxygenated in a separate vessel, and added to the medium in the ratio of 1 cc. of packed cells to 5 cc. of

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the medium. A similar proportion of red blood cells was added to the thermobarometers in these experiments.

RESULTS. (a) *Lack of influence of NaCl concentration on the Q_{O_2} of rat diaphragm, when the gas phase was 100 per cent oxygen, and in the absence of red blood cells.* Table 1 clearly shows that under the conditions commonly used for in vitro work, the concentration of sodium chloride in the medium (within reasonable limits) has little or no effect on the Q_{O_2}. It therefore seemed possible that the definite effects of NaCl seen in our previous in vivo work resulted from an exchange of ions between the blood serum and the tissue cells, which for some reason or other, did not occur under our in vitro conditions.

(b) *The influence of in vivo NaCl depletion on the Q_{O_2} of rat diaphragm, gas phase = 100 per cent oxygen, red blood cells absent.* In view of the above results

TABLE 1

Comparison of the Q_{O_2} of portions of the same rat diaphragms, showing the lack of influence of varying NaCl concentrations when the gas phase is 100 per cent oxygen and no red blood cells are present

CONDITIONS	Q_{O_2}		
	NaCl 100 m.e./liter	NaCl 150 m.e./liter	NaCl 200 m.e./liter
100 per cent oxygen without red blood cells	6.84	6.66	
	6.46	6.50	
	6.42	6.36	
	6.29	6.19	
		6.05	6.10
		6.30	6.25
		6.40	6.32
		6.36	6.50
Average Q_{O_2}	6.50	6.35	6.39
Per cent difference	+2	0	+1

it became of interest to determine the Q_{O_2} of diaphragmatic muscle of rats which had been depleted of NaCl during life. Others had previously shown that the tissue of rats in adrenal insufficiency had a lowered oxygen uptake in vitro, while the tissues from adrenalectomized rats adequately treated with NaCl had normal Q_{O_2}'s (6) (7). It had also been shown that animals may be depleted of NaCl by injecting isotonic glucose intraperitoneally, and these animals suffer the same electrolyte and water changes in blood and muscle as occur in adrenal insufficiency (8) (9). We employed the latter technique to obtain NaCl depleted rats. By trial and error it was found that the necessary depletion, in 60 to 90 gram rats, resulted from the intraperitoneal injection of about 20 cc. of 5 per cent glucose in water. The accumulated fluid was withdrawn from the peritoneal cavity at the end of four hours. The animals were placed in a cage with free access to water but no food, and sacrificed for the experiment 8 hours thereafter.

TABLE 2

The influence of NaCl concentration on the Q_{O_2} of rat diaphragm in the presence of blood cells, gas phase = air, and the lack of influence of NaCl when oxygen is substituted for air

Portions of the same rat diaphragm are compared in each case

CONDITIONS	Q_{O_2}		
	NaCl 100 m.e./liter	NaCl 150 m.e./liter	NaCl 200 m.e./liter
Air and red blood cells	4.20	6.65	
	4.19	5.19	
	4.57	5.95	
	4.32	5.75	
	4.34	6.06	
	5.90	6.48	
	4.09	5.99	
	3.92	6.01	
	4.63	5.92	
	4.74	5.87	
		6.28	7.54
		5.97	6.71
		5.74	6.81
		5.77	6.70
		5.86	6.67
Average	4.49	5.96	6.89
Per cent difference	−25	0	+16
100 per cent oxygen and red blood cells	7.94	7.44	
	6.92	6.85	
	8.10	8.76	
	7.80	7.87	
		7.09	7.54
		6.16	6.48
		6.01	6.15
		7.85	8.36
		8.43	8.23
Average	7.69	7.49	7.35
Per cent difference	+3	0	−2

TABLE 3

The lack of influence of osmotic pressure on the Q_{O_2} of rat diaphragm in the presence of red blood cells, gas phase = air or oxygen

Portions of the same rat diaphragm are compared in each case

CONDITIONS	Q_{O_2}		
	NaCl—150 m.e./liter Sucrose—75 m.e./liter	NaCl 150 m.e./liter	NaCl—150 m.e./liter Glucose—75 m.e./liter
Air and red blood cells	5.39	5.55	
	5.90	5.99	
	5.66	5.59	
	5.98	5.88	
	5.79	6.06	
		5.99	6.19
		5.69	5.58
		6.20	6.68
		5.89	5.90
Average	5.81	5.77	5.87
Per cent difference	+1	0	+1
100 per cent oxygen and red blood cells	8.10	7.30	
	7.32	7.36	
	7.80	7.19	
	6.78	6.79	
		7.59	7.79
		5.58	5.69
		8.20	8.78
		7.19	6.90
Average	7.16	7.15	7.29
Per cent difference	0	0	+2

The Q_{O_2}'s of the diaphragms of these rats were determined at the physiologically normal concentration of 150 m.e./liter of NaCl.

The oxygen consumptions of the diaphragms of three such animals, done in

duplicate, were as follows: 4.51, 5.08, Av. = 4.79; 5.69, 5.59, Av. = 5.64; 5.36, 5.35, Av. = 5.36. These values are significantly lower than the Q_{O_2}'s of normal rats at the same NaCl concentration, as shown in table 1.

These results seemed to confirm the difference between the living animal and tissue in vitro as regards the effect of varying the NaCl concentration, but offered no explanation. It occurred to us that another difference between the two sets of conditions was the presence of red blood cells in the living system and their absence in vitro.

(c) *The influence of NaCl concentration on the Q_{O_2} of rat diaphragm in the presence of red blood cells, gas phase = air.* The data in table 2 show that when in vivo conditions are more closely approximated (in the presence of red blood cells, gas phase = air) the influence of NaCl concentration on the Q_{O_2} of isolated muscle can be demonstrated. The effect of the NaCl is apparently exerted on the tranfer of oxygen from the red blood cells to the acceptors in the muscle, for when 100 per cent oxygen is substituted for air, the NaCl concentration loses its effect. This explains why the influence of NaCl was so readily demonstrable in the living organism, but did not manifest itself under the ordinary conditions of in vitro work. The amount of oxygen dissolved in the medium, when it is in contact with 100 per cent oxygen, must be at or above the maximum which obtains in the blood plasma of the living organism.

The mechanism by which NaCl influences the transfer of oxygen from red blood cells to tissue is somewhat clarified by other of our findings. The altered osmotic pressure of the medium does not appear to be a factor since equi-osmotic amounts of glucose or sucrose were without significant influence (table 3). However, it was found that a solution of hemoglobin, obtained by laking red blood cells and discarding the cellular elements, could substitute for red blood cells in demonstrating the influence of NaCl. For example, the differences in Q_{O_2} between NaCl concentrations of 100 and 150 m.e./liter in the presence of hemoglobin, gas phase = air, were as follows: 4.09 vs. 5.66, 4.51 vs. 5.68, 4.55 vs. 5.89, 4.90 vs. 6.06, Av. = 4.51 vs. 5.82. It seems likely, therefore, that NaCl acts by influencing the rate of dissociation of oxygen from hemoglobin and hence its availability to the tissues. The influence of NaCl on the dissociation of oxyhemoglobin has been previously demonstrated (10).

SUMMARY

The influence of the concentration of NaCl in the blood plasma upon oxygen consumption previously demonstrated in living animals, has been paralleled in vitro. The in vitro demonstration is possible only when the conditions more closely approximate the physiological than they ordinarily do. The effect is not apparent when tests are made in the absence of red blood cells or hemoglobin, and when the gas phase is 100 per cent oxygen. It is readily demonstrable in the presence of red blood cells or hemoglobin, and when the gas phase is air. Substituting oxygen for air in the latter case abolishes the effect. It is concluded that the NaCl acts both in vivo and in vitro (under physiological conditions),

by influencing the rate of dissociation of oxygen and hemoglobin, and thus affecting the amount of oxygen available for use by the tissues.

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SOME FACTORS AFFECTING AUGMENTATION OF PITUITARY GONADOTROPIC EXTRACTS BY HEME[1]

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The effect of the gonadotropic complex of the pituitary gland of certain animals is increased when it is injected with various substances which are themselves inactive in the stimulation of the ovaries of immature rats. These substances or augmenters include tannic acid (1), copper (2) and zinc salts (3, 4, 5), yeast extract and ash from yeast (2), male urine (6), blood serum (7), leucocytes (8), egg albumin (9), merthiolate (10) and chlorophyll (11, 12). Casida (13) found that the formed elements of blood augment gonadotropic extracts of sheep pituitary powder while McShan and Meyer (14, 15) separated the hemoglobin from the blood of the cow into heme and globin and found that the heme was an effective augmenter of the gonadotropic hormone of sheep, hog, man, cow and chicken pituitary glands.

These augmenters were usually tested by administering them with a basic dose of the gonadotropic extract which would when given alone stimulate the ovaries of immature rats measurably. As a rule the assays of pituitary gonadotropic extracts have been based on the administration of varying amounts over a constant period of time, and in testing for augmentation a known amount of the extract was combined and given with a known amount of the augmenter over the same period of time. However, in regard to the assay of gonadotropic extracts Fluhmann (16) gave a constant amount of sheep pituitary extract to immature rats over 5, 10, 15 and 20 days, and found that the ovarian weights were greater at 5 days than at 10, 15 and 20 days. Deanesly (17) found that when the injection period and amount of pituitary gonadotropic extract given to immature rats were varied the ovaries increased in weight during the first 10 days, were rather constant from 10 to 20 days, but decreased in weight during the 20 to 30 day period.

No systematic study has been made therefore of the ovarian weight response produced in immature rats by the pituitary gonadotropic complex when the amount of hormone, the period of administration and the age of animals are varied, and when under the same conditions the hormone is augmented by an effective augmenter such as heme. As far as we are aware no report has been made on the relationship of the fresh weight to the dry weight of the ovaries stimulated by the extract and those stimulated by the combination of the extract and augmenter, and the effect that corpora have on this fresh weight-dry

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weight relationship. This report is concerned with the presentation of the results obtained in the study of these factors and relationships.

An experiment was devised to study the effect of four different experimental factors upon the ovarian reaction of an unfractionated aqueous extract of pituitary. These factors were dosage of pituitary, presence or absence of a quantity of hemin[2] in the injection preparation, total number of injections, and age of the experimental rats. Four different dosages of pituitary extract were used: 3, 6, 12 and 24 mgm.-equivalent of acetone desiccated sheep pituitary per injection. One half of the rats received 0.5 mgm. hemin per injection mixed with the pituitary extract; the other half received no hemin. A comparison was made between rats 21 days of age and those 31 days of age at the beginning of the experiment. Five different numbers of injections were used, namely, 4, 8, 12, 16 and 20 (2, 4, 6, 8 and 10 days of injection).

TABLE 1

Average ovarian weight and percentage ovarian solids for pairs of rats on different treatments

MGM. PER INJ.		NO. OF INJS.									
		4		8		12		16		20	
		Age (days)									
Pit. extr.	Hemin	21	31	21	31	21	31	21	31	21	31
		Average ovarian weight, mgm.—Average percentage ovarian solids									
3	0.0	14–21.4	23–23.6	14–20.4	21–21.0	12–24.4	20–22.5	11–23.6	14–23.6	10–23.5	25–22.8
	0.5	22–19.0	25–21.4	36–15.6	38–19.8	38–15.8	52–21.8	82–14.9	47–21.4	34–17.6	150–20.8
6	0.0	20–20.4	25–20.7	21–20.8	54–18.0	22–21.0	74–18.6	30–22.1	40–21.0	22–21.9	58–21.7
	0.5	32–18.0	50–18.4	106–15.4	126–16.8	266–13.3	162–18.0	378–16.9	258–15.8	580–16.4	372–18.4
12	0.0	22–20.4	45–18.1	50–18.8	74–18.8	50–21.1	88–19.6	44–22.2	59–20.4	49–20.0	121–19.9
	0.5	48–17.4	56–17.0	143–15.8	190–16.4	355–15.6	346–16.4	434–16.6	589–17.0	474–18.6	698–17.8
24	0.0	34–17.4	60–17.0	78–18.2	134–17.9	84–21.2	152–20.3	64–21.5	101–20.0	68–20.8	107–19.6
	0.5	50–16.7	108–14.1	145–16.4	180–16.3	252–16.2	277–17.2	246–18.3	431–18.2	372–17.6	556–19.4

The animals were injected twice daily, in the morning and evening, with ½ cc. of the proper preparation and all injections were made subcutaneously. Autopsy was performed approximately 12 hours after the last injection. After the ovaries were removed and weighed, they were placed in a desiccator over sulphuric acid and left at room temperature until they came to a constant weight. From this treatment the percentage of solids was determined.

One hundred sixty rats (80 random pairs) were used in this experiment; no two pairs of rats received exactly the same treatment with respect to all four factors (table 1). In other words, all possible combinations of treatments were made. This meant that all dosages of pituitary were combined with all different

[2] The hemin was dissolved in weak sodium hydroxide, thus yielding "heme" which is the augmenting form of the substance.

numbers of injections for both age groups, with and without addition of hemin to the pituitary preparations. There was no intention of establishing the significance of the difference between any particular combination of treatments and any other particular combination. Only the more generalized effects of the factors under study were being sought.

It is not known definitely that the percentage of ovarian solids varies directly with the degree of luteinization. A determination was made of solids, however, in the belief that it gives an inverse quantitative indication of the degree of follicular stimulation, the degree of which ordinarily is estimated qualitatively. This belief is presumed from the usual lower percentage of solids in body fluids than in organ tissues.

The experiment was of such a nature that the results could be studied by analysis of variance and the data will be presented from the point of view of such an analysis.

Experimental results and discussion. Casual inspection of the data (table 1) would lead one to suspect that all four of the factors being studied were a source of variation both in ovarian weight and percentage ovarian solids. Calculations of the over-all range in results from the different treatments show the average ovarian weight for the 80 rats which received pituitary extract alone was 50 mgm. The addition to the extract of 0.5 mgm. hemin per injection to 80 other rats similarly treated in other respects produced 220 mgm. ovaries. The hemin-treated rats, therefore, showed ovarian weight approximately 440 per cent of the weight of those rats treated with pituitary alone. The 40 rats on each of the 3, 6, 12 and 24 mgm. dosages of pituitary, irrespective of other treatments combined with them, showed average ovarian weights of 34, 135, 196 and 154 mgm. In the experiment as a whole, therefore, there was a tendency for ovarian weight to reach a plateau at the 12 mgm. dosage level. Ovarian weights of 40, 88, 138, 176 and 232 mgm. were shown by respective groups of 32 rats on the 4, 8, 12, 16 and 20 injections. Rather uniform increases of ovarian weight occurred with the increase in number of injections throughout. The averages for the 21-day and 31-day rats showed 120 and 150 mgm. ovaries, respectively. Statistically, the probability of the variation associated with above factors, being due to chance alone was less than 0.01 (table 2—Hemin, AP, Inj., Age).

Hemin, pituitary dosage, and number of injections all produced a marked effect upon the percentage of ovarian solids. Age, however, showed an effect which was only on the margin of significance (table 2). The percentage of solids in the ovaries from rats treated with pituitary alone was calculated to be 20.6. This is to be compared with 17.4, the average percentage of solids in the ovaries of rats receiving both pituitary and hemin and a difference of 3.2 per cent. The percentages of solids for the different pituitary dosages were 20.8, 18.6, 18.4 and 18.2, respectively, for the 3, 6, 12 and 24 mgm. levels. The three higher dosages showed from 2.2 to 2.6 per cent less solids in the ovaries than the 3 mgm. dosage. The solids showed a decline from 18.8 to 17.9 per cent between the 4 and 8 injection treatment but there was an increase to 19.0, 19.6 and 19.8, respectively,

for 12, 16 and 20 injections. As stated above, the effect of age was questionable, the percentage solids for the 21-day old rats being 18.8 and for the 31-day, 19.2

Within the limits of the different variables studied in this experiment, the variation in terms of mean square (table 2) which was produced by hemin exceeded that produced by any other one factor. This was true both for ovarian weight and for the percentage of ovarian solids (table 2).

TABLE 2

Analysis of variance in ovarian characters

CAUSE OF VARIATION	D/F	OVARIAN WEIGHT		PERCENTAGE OVARIAN SOLIDS	
		Mean square	F	Mean square	F
Hemin	1	1,145,146	647.0*	431.98	332.3*
AP	3	205,722	116.2*	54.83	42.2*
Inj	4	178,123	100.6*	17.82	13.7*
Age	1	37,393	21.1*	5.07	3.9†
Hemin x AP	3	97,699	55.2*	5.47	4.2*
Hemin x inj	4	148,281	83.8*	8.30	6.4*
Hemin x age	1	140	0.08‡	54.64	42.0*
AP x inj	12	16,880	9.5*	4.82	3.7*
AP x age	3	20,510	11.6*	16.74	12.9*
Inj. x age	4	2,503	1.4‡	0.80	0.6‡
Hemin x AP x inj	12	13,460	7.6*	0.90	0.7‡
Hemin x inj. x age	4	2,614	1.5‡	5.84	4.5*
Hemin x AP x age	3	14,263	8.1*	6.50	5.0*
AP x inj. x age	12	4,211	2.38†	1.35	1.0‡
Hemin x AP x inj. x age	12	3,971	2.24†	2.00	1.5‡
Error	80	1,770		1.30	
Total	159	25,957		6.83	

Hemin = Presence and absence of hemin.
AP = Different dosages of pituitary extract.
Inj. = Different numbers of injections administered.
Age = 21 and 31 day old rats.

* = P <0.01.
† = P 0.01–0.05.
‡ = P >0.05.

The various interactions in which hemin is involved (table 2) indicate the effects of the other three factors, alone or in combination, upon hemin action, i.e., upon augmentation in milligrams of ovarian weight or upon decline in percentage ovarian solids.

The effects of number of injections and pituitary dosage upon action of hemin were about equal in causing variation both in augmentation of ovarian weight and decline in percentage of ovarian solids (table 2 and fig. 1). Age, however, in the experiment as a whole, modified the hemin effect only with respect to ovarian solids, where, as a matter of contrast, it was more effective than either pituitary dosage or number of injections.

The effects of variation in pituitary dosage upon augmentation in ovarian weight were modified about equally by age differences and differences in the number of injections. The results of differences in the number of injections on the other hand were affected by variations in pituitary dosage but not by differences in age. Age, however, although not capable of modifying augmentation in the experiment as a whole, did affect differentially the augmentation on different pituitary dosages. This was not true so far as different numbers of injections were concerned.

As stated above, age proved to be the most effective agent in modifying the effects of hemin upon ovarian solids. The effects of age, however, were in turn modified by the numbers of injections and by the pituitary dosage. If we now look at the variation in ovarian solids due to hemin but from the standpoint of the pituitary dosage, we find that it in turn is modified by variations in age but

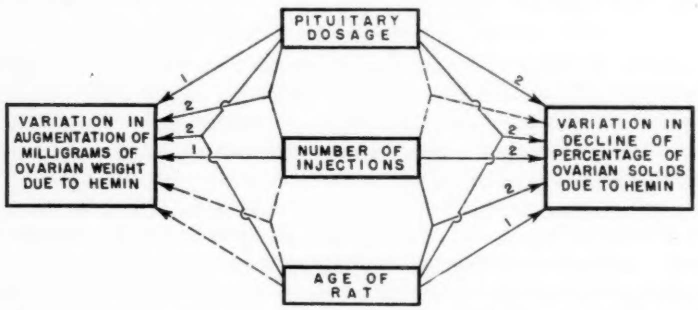

Fig. 1. Cause of variation in action of hemin upon ovarian characters. The broken arrows indicate that no significant effect of the factor was found. The branched arrows represent interaction of two factors. The numbers on the arrows indicate their rank in importance for causing variation; this ranking is based on the mean squares from table 2. A rank of 1 indicates a more important cause of variation than a rank of 2. All factors given the same rank were approximately equal in importance.

not by variations in numbers of injections. Again viewing it from the number of injections, we find that it was modified by age but not by pituitary dosage.

Augmentation in excess of 500 mgm. of ovarian weight has occurred on several different treatments within this experiment. The largest pair of ovaries obtained weighed 730 mgm. and this was in a rat 32 days of age at the time of autopsy, which had been treated for 10 days. It is believed that this degree of stimulation of the ovary is in excess of any previously reported for rats below the age of puberty.

In general, factors of an unknown sort appear to limit the attainable ovarian weight from injection of pituitary extract alone. This seems to be true whether increases in dosage are made with a constant treatment-interval or with an increasing treatment-interval. The addition of hemin to pituitary extract raises the limits for ovarian weight whether duration of treatment is held constant or is increased. The data emphasize the relatively low efficiency realized from unfractionated extracts alone and also point out that the ovary is capable of much greater reaction than is generally appreciated.

It has been shown (manuscript in preparation) that the percentage of ovarian solids is correlated with augmentation in ovarian weight (−0.70). The authors have developed the impression that there is a negative association between the percentage of ovarian solids and the degree of luteinization of the ovary. Methods of gathering objective data on the latter have not been devised, however, so the impression is a subjective one. The data support the statement, though, that hemin increases the water content of the ovary both on an absolute and a percentage basis. It is believed that this implies a modification of the physiological stimulation exerted on the ovary by the pituitary preparation. The balance between the follicle-stimulating and the luteinizing activities would appear to be altered by hemin. Greater emphasis seems to be exerted upon fluid storage in the follicles and less upon luteinization by the interacting hemin-pituitary preparation than by pituitary extract alone.

SUMMARY

The addition of hemin to pituitary gonadotropic extracts produced augmentation of ovarian weight and a decline in percentage of ovarian solids. The ability of three factors: age of test animal, dosage of pituitary extract and number of injections to cause variation in these effects of hemin was studied in an experiment involving 160 immature rats.

Variation in augmentation of ovarian weight was caused by: 1. Pituitary dosage. 2. Number of injections. 3. Pituitary dosage interacting with number of injections. 4. Pituitary dosage interacting with age of rat.

Variation in decline of percentage of ovarian solids was caused by: 1. Pituitary dosage. 2. Number of injections. 3. Age of rat. 4. Age of rat interacting with pituitary dosage. 5. Age of rat interacting with number of injections.

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RESPIRATORY CHANGES IN PULMONARY VASCULAR CAPACITY

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In motion picture studies in which respiratory changes in the output of the right and left ventricles were estimated separately in dogs with the chest closed, Shuler, Ensor, Gunning, Moss and Johnson (1942) found that during inspiration the output of the right ventricle increased, while the output of the left ventricle decreased. These inspiratory changes (which were reversed in expiration) were interpreted as due to *a*, an aspiration of blood into the right ventricle, and *b*, an increase in the pulmonary vascular capacity such that the filling and stroke volume of the left ventricle decreased.

The experiments here reported were designed to test the validity of these interpretations under somewhat simplified conditions using isolated terrapin hearts and terrapin heart-lung preparations perfused with Ringer's solution. The preparations were placed in a closed chamber representing the mammalian thorax, in which the pressure could be changed in a manner simulating the mammalian respiratory movements (see table 1).

In one series of experiments the terrapin heart alone was perfused, to simulate the right chambers of the mammalian heart, and its output measured by counting drops pumped from the aorta. When the heart was subjected to a negative pressure of one and a half inches of water (duplicating inspiration) the ventricular filling and stroke volumes increased two- to three-fold. This procedure resembled the inspiratory aspiration of blood into the mammalian right ventricle and the consequent increased output into the pulmonary artery.

In a second series the ventricle plus the lungs on its arterial side were perfused to simulate the right heart chambers plus the lungs of the mammal, and the blood flow from the pulmonary vein was measured. The flow decreased during "inspiration," showing that the increased capacity of the pulmonary vessels exceeded the increased output of the ventricle. This corresponds to a pooling of blood in the pulmonary bed in mammals during inspiration, so that the flow of blood into the left ventricle decreases even though the flow into the lungs increases.

In a third series the heart plus the lungs on its venous side were perfused to simulate the mammalian lungs and left heart chambers. In this series the output of the heart into the aorta fell when the "intrathoracic" pressure was lowered, corresponding to the lowered output of the mammalian left ventricle during inspiration. In both cases the lowered output results from decreased diastolic filling which, in turn, is a consequence of an increased fluid retention in the larger vascular bed of the lungs.

Thus, the various respiratory mechanical effects on blood flow in the mammalian chest were analyzed stepwise in three simple experiments.

In the light of these qualititative findings it seemed desirable to attempt to quantitate the respiratory changes in the capacity of the mammalian lung vessels, and to correlate them with the cyclic respiratory fluctuations in right and left ventricular output. Such observations should reveal whether or not the increased capacity of the pulmonary vessels in inspiration is sufficient to accommodate the extra blood pumped by the right ventricle in that phase of respiration.

The excised lungs of dogs were placed in a closed chamber representing the thorax. The trachea communicated with the exterior, and the pulmonary vessels, filled with blood treated with chlorozol fast pink to prevent coagulation, communicated with calibrated glass cylinders placed nearly horizontally at a height of six inches above the hilum of the lungs. The lungs were inflated by a negative "intrathoracic" pressure of four inches of water to simulate normal inspiration, and deflated by a return to negative one inch of water pressure to simulate normal expiration. By means of changes in the meniscus of the blood in the calibrated cylinders the volume changes in the pulmonary vessels were measured.

TABLE 1

Summary of three series of experiments on terrapin hearts and heart-lung preparations, with analogies in the mammalian circulation, and the results obtained

SERIES NO.	TERRAPIN PREPARATION PLACED IN A CLOSED CHAMBER ("THORAX") AND SUBJECTED TO NEGATIVE PRESSURES ("INSPIRATION")	PREPARATION SIMULATES IN THE MAMMALIAN CIRCULATION:	FLOW IN PREPARATION MEASURED FROM:	RESEMBLES FLOW IN MAMMALIAN CIRCULATION INTO:	EFFECT PRODUCED BY LOWERING THE PRESSURE ("INSPIRATION")
1	Heart alone	Right heart	Aorta	Pulmonary artery	Increase
2	Heart, plus lungs on arterial side	Right heart and lungs	Pulmonary vein	Left heart	Decrease
3	Lungs (on venous side) plus heart	Lungs and left heart	Aorta	Aorta	Decrease

For a ten kilogram dog these changes in pulmonary vascular capacity were 20 to 29 cc. (average 25 cc.) of blood in each inspiration or expiration. Attempts to measure these changes by other methods (Tigerstedt, 1903, 1907; Spehl and Desquin, 1909) have revealed similar volume changes.

Analysis[1] of the data published by Shuler and co-workers (1942) reveals that, in inspiration, the excess in output of the right ventricle over that of the left ventricle varies (in the respiratory cycles analyzed) between 18.7 cc. and 32.7 cc. in dogs weighing 10 kgm. and averages 27.1 cc., which closely approximates the

[1] This involves the assumptions that the average left or right ventricular stroke volume in dogs is 1.9 cc. per kgm. body weight (Henderson, 1923), and that the area changes measured by Shuler provide a means for estimating approximate volume changes.

inspiratory increase in pulmonary vascular capacity (averaging 25 cc. for a 10 kgm. dog) measured in the experiments here reported.

These observations indicate that the respiratory changes in pulmonary vascular capacity are approximately equal to the respiratory differences in the output of the right and left ventricles and lend further weight to the interpretations of Shuler and co-workers.

The changes in pulmonary vascular capacity are not apparent in the pulmonary arterioles and capillaries when these vessels are observed directly in the normally respiring mammal (Wearn, Ernstene, Bromer, Barr, German and Zschiesche, 1934). Therefore it seems likely that they occur mainly in the pulmonary veins, which are the largest pulmonary vessels, with thin distensible walls. Since Boyd and Patras (1941) have shown that even the relatively thick walled ventricles are affected by the intrathoracic pressure changes, it is likely that the pulmonary arteries also participate in the capacity changes.

Further analysis of the data of Shuler and co-workers (1942) indicates that the stroke volume of the right ventricle increased three to three and a half fold during inspiration, while the left ventricular stroke volume increased less than 50 per cent during expiration. These changes together with the changes in pulmonary vascular capacity clarify the concept of the respiratory action on the blood flow. Blood enters the lungs in increased volumes in inspiration at the time when the partial pressures of the gases in the blood differ most from those in the lungs, assuring efficient aeration. Blood leaves the lungs in a more nearly continuous flow, promoting a greater constancy of flow in the systemic vessels. If the changes in pulmonary vascular capacity occur largely in the pulmonary veins there is little if any inspiratory increase in the volume of blood in the capillaries where gaseous exchange occurs, yet there is apparently a greater volume flow through the lung capillaries during inspiration than during expiration so that efficiency of aeration is probably promoted.

SUMMARY AND CONCLUSIONS

1. Under simplified conditions, using perfused terrapin hearts and heart-lung preparations enclosed in an artificial thorax, the following effects of inspiration upon the mammalian heart were simulated:

a. Aspiration of blood into the right heart with a resulting increase in output.

b. An increased capacity of, and retention of blood in, the pulmonary vascular bed, reducing the flow of blood through the pulmonary vein.

c. A decreased filling and output of the left ventricle.

2. The changes in vascular capacity of excised dog's lungs accompanying inflation by an "intrathoracic" negative pressure of four inches of water and deflation by a return to atmospheric pressure were measured.

3. The magnitude of the increase in pulmonary vascular capacity in inspiration was found to be adequate to accommodate the extra blood pumped by the right ventricle in inspiration, as estimated from Shuler's results.

4. The respiratory changes in pulmonary vascular capacity probably occur chiefly in the veins.

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FURTHER STUDIES CONCERNING THE OVERT AND MASKED ACTIONS OF STEROIDS

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All hormonally active steroids examined up to the present time possess some degree of folliculoid activity (1–5). It has been postulated that certain common actions of steroids with otherwise vastly different pharmacological properties are due to the fact that they are all endowed with some folliculoid activity. Among the best studied folliculoid effects common to the steroid hormones are the ability to cause at least transitory vaginal cornification, uterine enlargement, mammary gland development, thymus involution, atrophy of the Leydig cells and the spermatogenic epithelium in the testis as well as to prevent the development of castration cells in gonadectomized rats. To mention only the most prominent representatives of the various groups of steroids one may say that these effects are exhibited by estradiol, testosterone, progesterone and desoxycorticosterone, but that the degree of this activity decreases in the order in which the compounds are mentioned. That all these apparently unrelated effects are really subordinate manifestations of a single (namely, the folliculoid) action, received further support by the observation that a striking parallelism exists between the degrees to which these effects are exhibited at the same time by any one compound. This theory has proved practically applicable inasmuch as it has frequently permitted the prediction of the degree to which a compound would exhibit the various folliculoid activities at a time when it had been examined for only one of these manifestations. Thus, for instance, a steroid having a pronounced anti-thymus effect invariably proved to be potent with regard to its anti-Leydig cell action and *vice versa* (6).

It may be asked that if all the above-mentioned effects are merely manifestations of a single pharmacological activity, how is it possible that in the case of certain compounds some actions (*e.g.* vaginal cornification, involution of the spermatogenic epithelium) are not manifest under all experimental conditions. We believe that this is due to the fact that some folliculoid effects are inhibitable and can be masked by other steroid hormone actions. The correctness of this assumption has been proven by actual experimentation since it was shown that the destruction of the spermatogenic epithelium (7) or the cornification of the vagina (8) normally elicited by estradiol or estrone is completely prevented if either progesterone or testosterone is administered simultaneously with the folliculoids. Since even the effects of added pure folliculoids are thus masked by luteoid and testoid compounds, it is hardly surprising that the slight folliculoid activity inherent in the testosterone or progesterone molecule can also be

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masked in this manner under certain experimental conditions. This further elaboration of our theory likewise proved of heuristic value inasmuch as it helped to explain the rather surprising fact that small doses of testosterone cause testis atrophy, while large doses tend to exhibit the opposite effect. It has been assumed, in agreement with the above considerations, that the production of testis atrophy is due to the folliculoid effect of testosterone which endows the compound with an anti-spermatogenic action. At low dose levels this effect is overt, while at high dose ranges it is masked by the independent spermatogenic action of the compound. The fact that depending upon the dose level one or the other of these two activities prevails, can readily be explained if we assume that the effective threshold for the spermatogenic action is much higher than that of the anti-spermatogenic action; hence at low dose levels the former cannot manifest itself. That this explanation is correct was shown by the administration of a mixture containing a constant proportion of Δ^5-androstene-3(β).17 (α)-diol and α-estradiol. The former compound is spermatogenic at all effective dose levels (apparently because it possesses comparatively slight folliculoid properties), while the latter substance is, of course, intensely anti-spermatogenic. As we predicted on the basis of our theory, this mixture proved to cause testis atrophy at a low, but not at a high dose level (9).

The experiments upon which we should like to report in this communication have been planned in the hope that our theory might also help to explain the rather contradictory results recorded in the literature concerning the production of vaginal cornification in spayed rodents with various androstane and alkyl-substituted androstane derivatives. With many of these compounds some workers obtained marked vaginal cornification at low dose levels, while others were unable to confirm such an "estrogenic" effect although much higher doses were used. It will be recalled that we (8) found that if small doses of estrone are administered simultaneously with large doses of progesterone the vagina cornifying effect of the former is inhibited by the latter and vaginal mucification results. In these experiments 400 γ of progesterone were required to inhibit the action of estrone. Subsequent investigators (10–13) essentially confirmed these observations but the ratio between estrone and progesterone necessary to inhibit the cornifying effect of the former differs greatly in these publications and it has not been possible to establish just how much progesterone is needed to mask this effect of a given dose of a folliculoid. According to our theory this is understandable since the effect depends not only upon the ratio between the two steroids, but also upon the dose level tested. At low dose ranges we would expect the folliculoid and at high dosage levels the luteoid action to prevail.

The following experiment shows that in this case prediction on the basis of our theory again received support from actual observations.

Twelve female albino rats weighing 160–175 grams were spayed and two days later divided into two groups of six animals. Group I received a mixture of 0.5 mgm. of progesterone and 25 γ of α-estradiol dissolved in 0.2 cc. of peanut oil per day. Group II was given 10 mgm. of progesterone and 500 γ of α-estradiol in 0.2 cc. of peanut oil per day. The daily dose was administered in two subcu-

taneous injections in both these groups, treatment being continued for ten days. The animals were sacrificed twenty-four hours after the last injection. In this manner group II received twenty times as much of the same hormone mixture as was given to group I. All other experimental conditions in the two groups were identical. Daily examination of the vaginal smears throughout the experimental period indicated that all animals of group I showed continuous estrus throughout the experiment, while those of group II revealed only transitory vaginal estrous changes during the first two days. At autopsy the uteri of group I were greatly dilated with fluid exhibiting a typical estrous appearance, while in group II the dilatation was much less evident. Thus it appears that at a low dose level our hormone mixture caused continuous estrus, while this was not the case at the high dose level.

Figure 1 is a schematic drawing which will help to visualize the interaction between the two hormones. The vaginal cornifying effect seen with increasing

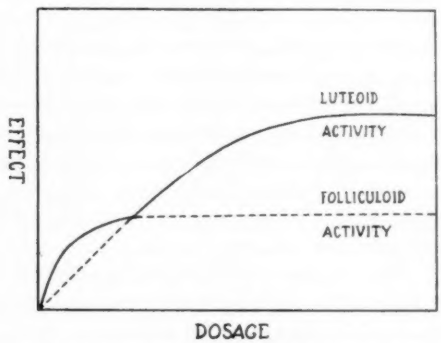

Fig. 1. Diagram showing manifest and masked activities of steroids. Solid line represents manifest and interrupted line masked activities.

doses of estradiol rapidly reaches a maximum above which it is not significantly enhanced by raising the dose. On the other hand the degree of progesterone response rises very slowly but continues to increase over a wider dose range. Hence at low dose levels the folliculoid and at high dose levels the luteoid action will be manifest. It may be said that the transitory vaginal estrus obtainable with all hormonally active steroids can also be explained on the basis of these observations since it must be assumed that the slight folliculoid activity inherent in such compounds as testosterone, progesterone and desoxycorticosterone could only be manifest at very low dose levels in the face of their strong inhibitory actions. Thus signs of vaginal stratification and cornification are detectable only during the first few days of treatment when the hormone concentration in the body is still low. It will be noted that our schematic drawing can equally well be used to illustrate either the effect of a single compound endowed with folliculoid and luteoid activity or of a mixture of a folliculoid and a luteoid compound. Depending upon the relative amounts of these two activities present in

a single steroid or a mixture, the point at which the two curves cross will shift to the left or right and the effective dose range of the folliculoid activity will become smaller or larger respectively.

In conclusion we wish to re-emphasize the broader implications of these observations concerning the interpretation of hormonal actions in general. It has often been claimed that if a crude extract of an endocrine gland can be separated into two fractions with qualitatively different effects, one may assume that two distinct hormones are present which have at least partially been separated. The above observations show that this type of argument is not conclusive since qualitatively different effects (cornification or mucification) may be obtained with a mixture containing a constant proportion of two hormones depending upon the dose level tested. It is essential, therefore, in the case of comparatively impure extracts to test the activity of each fraction over a wide dose range before claiming that a separation into distinct principles has been effected.

SUMMARY

Experiments on spayed rats indicate that a mixture containing a constant proportion of progesterone and α-estradiol causes vaginal cornification at low, but not at high dose levels. The experiments are taken to support the concept according to which the transitory vaginal cornification elicited in the spayed rodent by all hormonally active steroids is due to the fact that these compounds contain a certain amount of folliculoid activity. This is manifest during the first few days of treatment when the hormone concentration in the body is still low but becomes masked when this concentration rises above a certain level.

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RÔLE OF THE RENAL INNERVATION IN RENAL TUBULAR FUNCTION

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The data given here were obtained some time ago (1939) but their publication is considered worthwhile because they are evidently the first definite proof that the renal nerve-supply does not affect renal tubular secretion: at least with regard to water, chloride, and phenol-red.

The antecedant contributions, too numerous to be mentioned here, have shown or have failed to show differences in the rate of excretion of various substances between the denervated and the innervated kidney. In no instance, however, was the rate of filtration known; consequently the amount resorbed or the amount outwardly secreted, as the case may have been, was unknown. With the introduction of the inulin-clearance as a direct measure of glomerular filtration, simultaneously by Shannon (1934) and Richards, Westfall and Bott (1934), it has become possible to find the fraction of material resorbed or outwardly secreted. Incidentally, in the dog, the creatinine-clearance is equal to the inulin-clearance (Shannon, 1935a).

The amount of substance resorbed per unit time can be easily calculated:

$$\text{Amount resorbed} = \text{Amount filtered} - \text{Amount excreted}$$

The amount filtered equals the rate of filtration of plasma multiplied by the concentration of the free substance in the plasma, or, more strictly, in the plasma-water. All the Cl in the plasma is free. The amount of water filtered per unit time is taken equal to the rate of glomerular filtration (=inulin- or creatinine-clearance). This assumes that the volume occupied by the non-filterable constituents of the plasma is negligible.

For a substance which is outwardly secreted:

$$\text{Amount secreted} = \text{Amount excreted} - \text{Amount filtered}$$

Phenol-red is such a substance but the amount of it filtered was not known because the protein-bound fraction was not ascertained. The relative activity of the tubules of both kidneys with regard to PR-secretion could, however, be estimated by comparing the ratios of the inulin- and PR-clearances for both kidneys (table 1).

Chloride, water, and phenol-red were chosen as test-substances because they are easy to measure and are transported by actual activity on the part of the tubules (see Results).

To make sure of complete denervation, the test-kidney was excised and transplanted.

The surgery was performed by Dr. Kenneth W. Thompson, Department of

Surgery, Yale University. To Dr. Robert W. Clarke of the Laboratory of Physiology, Yale University, the writer owes his initiation into the technique of measuring renal clearances and thanks for help throughout.

METHODS. *1. Surgical.* The hound was a young female weighing 15.2 kgm. *Operation 1. Transplantation of right kidney to right femoral region* (Jan. 3, 1939). After all was prepared and the right ureter had been mobilised with plenty of fat to spare its blood-supply, the right renal pedicle was divided close to the aorta and the anastomosis done forthwith. In order to have approximately equal ischemia in both kidneys during the operation, the rubber-clamp on the left renal pedicle was tightened just after the right pedicle was divided. After the anastomosis, the clamp was removed from the femoral artery and the blood was allowed to flow through the kidney for 30 seconds. There was no leak at the junctions. The femoral and renal veins were then anastomosed, after which all clamps were immediately removed. The circulation became re-established apparently satisfactorily. The blood-supply had been obstructed for 25 to 30 minutes. The transplanted kidney was firmly sutured in place by tacking fatty tissue on all sides of it. After the operation 300 cc. saline plus 10 per cent glucose were given intravenously. Postoperative convalescence was uneventful. *Operation 2. Transplantation of central portion of bladder including trigone to ventral abdominal wall* (Feb. 2, 1939). The bladder was developed with special care to preserve the blood-supply of trigone and ureters. Neither ureter was dilated. The bladder was then opened and the distal end of the urethra was ligated. The mucous membrane of the bladder was united to the skin of the abdominal wall. Saline-glucose was again given intravenously and continued subcutaneously a few times during the following week. Convalescence was uneventful. The discharge of urine from the ureteral orifices was rhythmical throughout the period of study.

2. Convalescence and training. During the subsequent 2 days, the bladder and thigh-region were bathed twice daily with 85 per cent ethyl alcohol. Subsequently the bladder was washed twice daily with warm sterile saline and warm saturated boric acid. There were no signs of infection. The dog was constantly vigorous and had a good appetite, normal feces, and a rectal temperature of about 38.5°C. Except for the first day after the operation, it was fed milk and a pound of meat daily. It gradually increased to normal weight from the initial somewhat emaciated condition of January 3.

Tests during the first week after the operation showed the urine to be acid to litmus and alkaline to methyl-orange and methyl-red. There was, however, some pus in the urine from the right ureter; but the urine was never malodorous. A renal antiseptic was administered: 7.5 grains methyl-amine plus 7.5 grains NaH_2PO_4 (as an antibase) were given, with the meat, twice daily. On February 23, the pus was almost negligible and the antiseptic was no longer given. On January 10 glycosuria disappeared but there was always a slight amount of protein in the urine from the right ureter. Preliminary measurements of urinary flow, depression of the freezing-point, chlorine- and urea-excretion gave no evidence of malfunction in either kidney. The concentration of urea in the

blood was normal notwithstanding the meat diet. Phenol-red, injected subcutaneously, appeared simultaneously from both ureters (detected by application of $NaHCO_3$ to the exteriorised bladder).

The dog, which was good-tempered, was trained to lie quietly while strapped horizontally to the dog-board. An anesthetic was never used outside of the surgical operations.

3. Physiological procedure. In some experiments the inulin-clearance was used as a measure of glomerular filtration (table 1). The infusion-solution consisted of 40 grams inulin (Pfanstiehl, c.p., non-pyrogenic to dogs) dissolved at

TABLE 1

Two experiments showing amounts of water and chlorine resorbed per unit volume of glomerular filtrate and the relative potencies of both kidneys to excrete phenol-red

TIME	URINARY FLOW (cc./min.)		PLASMA (MGM. PER CENT)			URINE (MGM. PER CENT)				RATIO OF CLEARANCES (LEFT/RIGHT)		MGM. Cl RESORBED/100 CC. GLOM. FILTRATE		CC. WATER RESORBED/100 CC. GLOM. FILTRATE		MGM. Cl EXCRETED PER MIN.	
						Inulin		PR									
	Left	Right	Inulin	PR	Cl	Left	Right	Left	Right	Inulin	PR	Left	Right	Left	Right	Left	Right
min.																	
	Infusion-expt. with inulin and PR (Feb. 22, 1939)																
−30	About 300 cc. tepid tap-water by gastric tube																
−15	About 250 cc. tepid tap-water by gastric tube																
0	Infusion-solution enters left ext. jug. vein at a little over 5 cc./min.																
10	PR is evident from both ureters simultaneously ($NaHCO_3$-test)																
20	Rate of infusion lowered to 2.5 cc./min.																
25-37	0.6	1.1	337	1.42	390	9,350	6,500	44.0	33.8	0.79	0.71	384.5	377.6	96.4	94.8	0.87	2.6
43.5-56.5	0.185	0.63	361	1.52	390	14,000	11,750	90.5	74.5	0.32	0.36	383.9	377	97.4	97.2	0.44	2.9
97-110	0.78	1.09	380	1.64	390	17,200	8,250	117.0	58.3	1.49	1.44	385.3	373.5	97.8	95.4	1.71	3.9
	Infusion-expt. with inulin and PR (Mar. 1, 1939)																
−64	About 600 cc. tepid tap-water by gastric tube																
−37	About 500 cc. tepid tap-water by gastric tube																
−18	30 cc. priming solution (19 cc. 0.5 per cent PR; 30 cc. Ringer's; 2 g. inulin) subcutaneously; 15 cc. through each side of lateral abdominal wall																
0	Infusion-solution passes into left ext jug. vein at ca. 4 cc./min.																
10	Rate of infusion lowered to 2 cc./min																
19-25	2.58	1.58	170	1.25	355	1,600	1,750	16.9	20.2	1.49	1.37	353.9	352.1	89.3	90.3		
37-42.5	2.49	1.38	229	1.68	357	2,300	2,250	22.7	24.6	1.85	1.67	355	354	90.0	89.8		
71-81	1.44	1.16	294	1.83	364	2,875	2,750	27.6	28.8	1.31	1.19	361.9	360.8	89.8	89.3		
86-94.5	2.38	1.82	315	1.91	363	3,325	3,300	31.0	31.7	1.32	1.28	361.1	361.1	90.5	90.4		
106.5-116	2.3	1.43	336	1.96	363	3,775	3,550	33.7	33.2	1.72	1.64	362.5	361.9	91.1	90.5		

85°C. in mammalian Ringer's; to this was added 40 cc. 0.5 per cent PR. The water had been freshly glass-distilled and the solution was sterilised before use. The solution was passed through the left external jugular vein at 2.5 to 5.0 cc. per minute. In some instances a priming solution was given (table 1, Mar. 1). The urine was diluted immediately after being collected and measured.

Because we were studying tubular activity, the attempt was made to have the plasma-PR close to 1.5 mgm. per 100 cc. (table 1). At this concentration the fraction excreted by secretion approximates 83 per cent, but at a plasma-concentration of about 40 mgm. per 100 cc. only 35 per cent is excreted by secretion (Shannon, 1935b). In other instances the clearance of exogenous creatinine

was taken as the measure of glomerular filtration (table 1). Ten per cent solutions were injected subcutaneously into the lateral abdominal region: one-half of the total quantity into each side, after which the sides were briefly massaged. The creatinine was dissolved first in a minimal quantity of distilled water as it is not soluble in Ringer's to the extent of 10 per cent. The urine was collected into test-tubes by gentle suction (6–7 mm. Hg) applied to both ureteral

TABLE 2

Three experiments showing amounts of water and chlorine resorbed per unit volume of glomerular filtrate

TIME	URINARY FLOW (CC./MIN.)		PLASMA (MGM. PER CENT)		URINE (MGM. PER CENT) Creatinine		MGM. Cl RESORBED/100 CC. GLOM. FILTRATE		CC. WATER RESORBED/100 CC. GLOM. FILTRATE		MGM. Cl EXCRETED PER MIN.	
	Left	Right	Creat.	Cl	Left	Right	Left	Right	Left	Right	Left	Right
min.												
	(April 21)											
−41	25 cc. 10 per cent creatinine in Ringer's, subcutaneously											
−22-−7		0.14	14.5	435		1,431		427.5		99.0		1.04
0	About 900 cc. tepid tap-water by gastric tube											
19–47	0.047	0.127	18.4	407	2,890	2,270			99.4	99.2		
67–76	1.25	0.74	16.8	392	326	356	386.8	387.3	94.8	95.8	1.25	0.73
93–99	1.9	1.0	16.2	392	222	226	388.3	389.2	92.7	92.8	0.95	0.45
	(April 24)											
−54	30 cc. 10 per cent creatinine in Ringer's, subcutaneously											
−21-−6	0.059	0.133	21.5	420	4,320	2,475			99.5	99.1		
0	About 900 cc. tepid tap-water by gastric tube											
22–23	0.105	0.225	24.4	325	5,010	2,250	391.7	390.8	99.5	98.9	0.77	0.88
71–77	2.47	1.18	16.2	388	246	351	385.2	384.9	93.4	95.2	1.04	0.77
95–101	2.04	1.70	16.1	389	227	192	386.9	385.9	92.9	92.0	0.61	0.68
	(May 31)											
0	About 700 cc. tap-water by gastric tube											
25	25 cc. 10 per cent creatinine in Ringer's, subcutaneously											
88–93	3.3	1.8	12.4		147.4	143.2			91.6	91.3		
101–106	4.2	1.7	12.3		144.3	126.0			91.6	90.2		
115–120	3.2	1.7	12.0		179.0	161.0			93.0	92.5		

orifices simultaneously. The glass-tubes applied to the orifices were capillaries enlarged into small cups at one end.

4. Chemical methods. All analyses were made in duplicate. The values were compared with standard solutions. Blood-clotting was prevented by a minute amount of heparin in the needle and nozzle of the syringe.

a. *Urea* was estimated manometrically (Van Slyke, 1927). The urine was diluted 100 times.

b. *Chlorine* in plasma and urine was analyzed by the titrimetric method of Volhard (1878) as modified by Van Slyke and Sendroy, Jr. (1923).

c. *Inulin* in plasma and urine was measured, after yeast-adsorption and acid

hydrolysis (see Shannon and Smith, 1935), by the Shaffer-Hartmann-Somogyi method (see Shaffer and Somogyi, 1933) using Shaffer-Hartmann reagent "50" and Somogyi's (1931) procedure for deproteinisation of the plasma.

d. *Exogenous + endogenous (?) creatinine* was analyzed by the Jaffé reaction (method of Folin and Wu, 1919; see also Shannon *et al.*, 1932). The plasma was made protein-free by an equal volume of 6 per cent Na_2WO_4 and of 0.33N H_2SO_4. The urine was diluted 100 to 3000 times so that differences in the colorimetric readings (Duboscq colorimeter supplied with green Wratten filter) were not over 50 per cent and generally much less than that. In some experiments an Evelyn photoelectric colorimeter was used but no distinct advantage was so gained.

e. *Phenol-red* was measured colorimetrically. One drop of saturated Na_2CO_3 was added to the 2 cc. of plasma or the tenfold diluted urine just before taking a reading. From this reading the value of the plasma, collected before administration of the PR, was subtracted. An ε 74 Wratten filter was used with the Duboscq colorimeter (see Shannon, 1935a).

Results. Since the urine of the mammal is ordinarily hypertonic to the blood (Hoppe-Seyler, 1859), the tubular resorption of water beyond the stage producing isosmoticity with the blood must be an active process. Even in ordinary diuresis, the mammalian urine is markedly hypertonic. Furthermore, when the Cl-concentration of the blood falls, the Cl-concentration of the urine may fall, not only well below the concentration of Cl in the plasma (see tables 1 and 2), but almost to the vanishing point. Clearly, Cl must be actively resorbed by the tubules. Lastly, the fact that the PR-clearance invariably exceeds the inulin-clearance (table 2) shows that PR is secreted outwardly by the tubules.

Inspection of tables 1 and 2 shows that the amount of water and chlorine resorbed *per unit volume of glomerular filtrate* is identical in the transplanted and in the non-transplanted kidney. This is true even when a large fraction of this resorption is the result of tubular activity. Furthermore, the ratios of the inulin- and PR-clearances are practically identical for both kidneys (table 1) showing that the fraction of phenol-red outwardly secreted by the tubules of both kidneys is the same. These data demonstrate, conclusively in our opinion, that the renal nerve-supply has no influence on renal tubular activity, at least with regard to water, chloride, and a foreign substance such as phenol-red. Because the active transport of substances by the tubules follows the mass-law—at least with regard to substances which have been most extensively studied with this respect (Shannon, 1939)—one would not expect, even *a priori*, the renal nerves to exert an influence on tubular secretion.

On the other hand, our data show, as do those of many of our predecessors, that the rate of urinary flow and of the excretion of chloride is not necessarily identical from both kidneys. As shown in this paper, however, such differences do not reside in dissimilarities in tubular function and must, therefore, be due to differences in glomerular activity. We do not, however, imply that changes in glomerular activity are necessarily concomitant with renal denervation.

In the above experiments the animal was not anesthetised. It was so used

to being strapped on the dog-board and having an infusion-needle in its external jugular vein that it frequently yawned during the procedure.

SUMMARY AND CONCLUSION

1. A dog's kidney was successfully transplanted to the femoral region by end-to-end anastomosis of the renal vessels with the femoral; the bladder and ureteral orifices were exteriorised.

2. The amounts of water and of chlorine resorbed per unit volume of glomerular filtrate (inulin- or creatinine-clearances) were identical in both the transplanted and non-transplanted kidney. This was true both during antidiuretic and diuretic urinary flows.

3. The ratios of the inulin- and phenol-red clearances were practically identical for both kidneys, showing that the fraction of phenol-red outwardly secreted by the tubules of both kidneys was the same.

4. Our data demonstrate that any differences in the rate of excretion of water and of various solutes by the normal and fully denervated kidney are not due to dissimilaritites in tubular function and hence must be due to differences in glomerular circulation.

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A DIURNAL RHYTHM IN THE BLOOD SUGAR OF THE WHITE RAT

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It has been observed by Cori and Cori (2) that the concentration of blood sugar is an important factor determining the rate of glycogen formation in the liver. We also note that those investigators who have more recently studied diurnal variations of liver glycogen in the white rat (3, 7, 8) present convincing evidence that a definite diurnal rhythm of glycogen deposition occurs in this animal and that this rhythm is due to cyclic feeding habits. The natural corollary to these two observations is that the white rat should show a diurnal rhythm also in blood sugar concentration, and that this rhythm, like that of liver glycogen, should be determined by cyclic feeding habits. The purpose of the investigation reported here was to test this hypothesis.

Aside from clinical observations on the diabetic human being studies on diurnal variations of blood sugar concentration are rare. The few authors who have attacked the problem have worked on a variety of animals with a consequent variety of results. Krasnjanskij (10) working on normal human subjects found a sharp rise in blood sugar following each meal. However, his data give no suggestion of any general diurnal trend. Allcroft (1) found a different situation in the lactating cow. He was able to demonstrate a diurnal rhythm with the maximum blood sugar concentration occurring at 3 to 4 a.m. and the minimum at 8 to 9 a.m. He was unable to find any such rhythm in the dry cow or sheep. Euler and Holmquist (4) working on the rabbit, found a definite blood sugar rhythm with the maximum occurring between 4 and 10 a.m. Still later Jores (9) working on ten normal human subjects disagreed with Krasnjanskij to the extent that he was able to demonstrate a diurnal rhythm in the blood sugar with the maximum concentration occurring at 4 a.m. and the minimum at 3 to 4 p.m.

METHODS. Virgin female rats four to six months of age and 175 to 250 grams weight were used in all experiments. In the present work a certain importance is attached to the diurnal spontaneous activity rhythms of the animals used and the fact that the spontaneous activity rhythm of the female rat is clearer and less variable than that of the male accounts for the exclusive use of females in these experiments. It may be pointed out, however, that experiments on male rats indicate that similar results are obtained with both sexes. The rats were kept in wire cages with activity recorders of the type illustrated and described by Griffith and Farris (5) and were exposed to normal daylight and darkness. Water was always available and Purina rat food was used consistently.

The blood sugar concentrations were determined by the microtitration method

of Miller and Van Slyke (11). This method gives essentially the fermentable sugar thus yielding values lower than those obtained by methods which determine total reducing substances. The blood was obtained by clipping the tip of the animal's tail with a pair of scissors. Three or four drops were allowed to fall into a waxed dish containing powdered sodium citrate to prevent coagulation. From this dish the 0.1 cc. required was pipetted out and precipitated in cadmium sulfate solution. It was observed that blood sugar values were abnormally high when more than three minutes elapsed between the time an animal was picked up and the time the last drop was obtained from the tip of its tail. This was probably an excitation effect akin to Cannon's "emergency hyperglycemia." Consequently, all the determinations used in this paper are based on blood samples obtained in less than three minutes.

All blood samples were taken at 12 noon and 12 midnight (EST) $\pm$ 45 minutes. It was observed that when a rat was bled at twelve hour intervals for a number of days, the blood sugar values obtained from the later bleedings frequently showed great variability, deviating markedly from the pattern obtained in the first few bleedings. A possible explanation of this is that frequent clipping of the tail caused painful swelling, a disturbing condition which was reflected in the blood sugar. Consequently, the rats used in these experiments were never bled more than three consecutive times at twelve hour intervals.

Procedure and results. The first point to be investigated was whether or not the blood sugar of the normal white rat, feeding freely, exhibits a diurnal rhythm. This was investigated by the simple procedure of making a series of blood sugar determinations on blood samples taken from such rats at both noon and midnight. In this experiment fifty-four determinations were made on eleven different animals. The animals were allowed a three to five day recovery period after each set of three consecutive bleedings before being used again. Half of the bleedings were in the order noon-midnight-noon while the other half were made in the opposite order, midnight-noon-midnight. This procedure was followed in all the experiments described and was intended to elininate the possibility that the observed changes in blood sugar concentration might be a direct response to the experimental technique used. A true diurnal rhythm obviously would give reversed patterns under the two different procedures whereas a direct response to the technique of bleeding would be the same in both cases.

The results of these experiments are given in table 1. The data in this table make it obvious that the blood sugar concentration of normal animals is approximately 10 per cent higher at midnight than at noon. The usual criterion for statistical significance, i.e., that the difference between the mean midnight value and the mean noon value must be more than twice its standard error, is used throughout this paper. In this case the difference between the means is more than six times its standard error.

Having established the presence of a diurnal blood sugar rhythm by this first group of experiments on animals feeding freely, it then became desirable to know to what extent this rhythm was affected by the animals' cyclic feeding

habits. Two procedures were used to obtain this information, i.e., fasting and enforced reversal of the animal's feeding cycle. The fasting studies were made upon two groups of six rats each. They were subjected to the following fasts.

Group I: *a.* Thirty-six hour fast beginning at midnight, the first blood sample being taken at noon twelve hours after the removal of food.

b. Forty-eight hour fast beginning at noon, the first blood sample being taken at noon twenty-four hours after the removal of food.

c. Forty-eight hour fast beginning at midnight, the first blood sample being taken at midnight twenty-four hours after the removal of food.

TABLE 1

Determinations on rats allowed to feed freely

VALUES	NUMBER OF ANIMAL										
	14	15	16	17	18	19	20	21	22	23	25
mgm. per cent											
Day	96	93	98	96	99	94	99	89	94	99	97
	97	97	99	88	97	93	101	90	88	97	106
					92			99		92	
					97					100	
					100					94	
					102						
Night	104	113	123	98	105	104	109	103	103	108	119
	105	96	107	99	106			110		104	
					103					97	
					104					106	
					104						
					110						

	Standard deviation	Mean	Standard error of mean
Day values	4.24	96.1	0.78
Night values	6.23	105.9	1.27
Difference between means		9.8	
Standard error of difference between means			1.49

Group II: *a.* Fast corresponding to *c* above.

b. Sixty hour fast beginning at midnight, the first blood sample being taken at noon thirty-six hours after the removal of food.

The statistical summaries of the results of these fasting experiments are to be found in part I of table 2. A study of the table makes it clear that the diurnal rhythm in blood sugar concentration persists during as much as forty-eight hours of fasting but disappears in fasts longer than this. As the length of the period of fasting is increased, the curve of diurnal variation tends to flatten out. After about forty-eight hours of fasting it levels off at approximately 80 mgm. per cent which is roughly 10 mgm. per cent below the average noon figure for animals allowed to feed freely.

It is of interest to note that in the first group of animals a fast of forty-eight

TABLE 2

Statistical summaries of experiments involving fasting and reversal of feeding cycle

CONDITIONS OF EXPERIMENT	TIME OF BLEEDING	STANDARD DEVIATION	MEANS	ST. ERROR OF MEANS	ST. ERROR OF DIFFERENCE BETWEEN MEANS	IS THE DIFFERENCE SIGNIFICANT?
			mgm. per cent			
I. Fasting experiments:						
A. Group I						
1. Thirty-six hour fast beginning at midnight, the first blood sample being taken at noon 12 hrs. after food removal	N*	4.04	79.8	1.17		
	M*	6.88	85.1	2.81		
			5.3†		1.49	Yes
2. Forty-eight hour fast beginning at noon, the first blood sample taken at noon 24 hrs. after food removal	N	5.34	78.7	1.88		
	M	9.64	90.2	4.31		
			11.5†		4.71	Yes
3. Forty-eight hour fast beginning at midnight, the first blood sample being taken at midnight 24 hrs. after food removal	N	5.34	86.0	2.39		
	M	8.40	84.8	2.66		
			1.2*†		3.58	No
B. Group II						
1. Fast corresponding to "3" of group I (fig. 1)	N	5.64	86.8	2.30		
	M	7.27	99.0	3.46		
			12.2†		4.16	Yes
2. Sixty hour fast beginning at midnight, the first blood sample being taken at noon 36 hrs. after food removal (fig. 2)	N	6.90	76.4	1.99		
	M	3.65	79.8	1.49		
			3.4†		2.48	No
II. Experiments on rats allowed to feed only from 9 a.m. to 5 p.m.						
A. Determinations on the 5th and 6th days of the new feeding regimen (fig. 3)	N	5.71	94.8	1.80		
	M	5.40	102.0	2.23		
			7.2†		2.86	Yes
B. Determinations on 14th to 15th days of the new feeding regimen (fig. 4)	N	3.94	105.3	1.61		
	M	4.75	95.0	1.37		
			10.3†		2.12	Yes
C. Determinations on the 20th and 21st days of the new feeding regimen	N	6.36	109.8	1.76		
	M	6.57	97.3	2.49		
			12.5†		3.05	Yes

* N = noon, M = midnight.

† Difference.

hours beginning at noon did not abolish the diurnal rhythm whereas a forty-eight hour fast beginning at midnight did so. A possible explanation of this is that since rats normally do not begin to feed until 6 or 7 p.m., a fast beginning at noon does not become effective until six or seven hours later, whereas one beginning at midnight becomes effective immediately. Hence, a forty-eight hour fast beginning at noon is, physiologically speaking, only a forty-one or forty-two hour fast. It is also to be noted that the diurnal rhythm was more easily abolished in the first group of animals than in the second, a sixty hour fast being required in the latter case. The only notable difference between the two groups is that the rats of the first group (18 to 21, 23 and 25) were from one to one and a half months older than those of the second group and had been

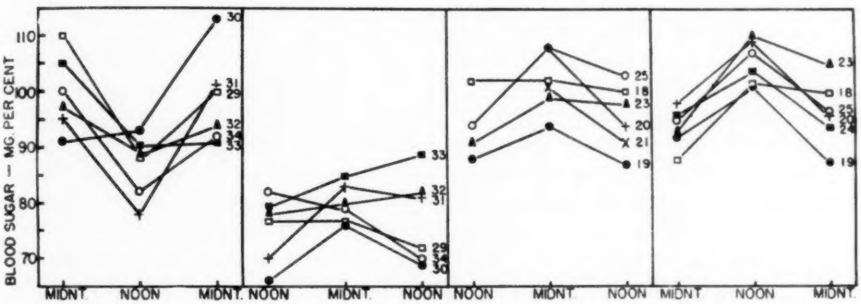

Fig. 1 Fig. 2 Fig. 3 Fig. 4

Fig. 1. Determinations on rats which fasted a total of 48 hours. Food was removed 24 hours before the first midnight determinations. These rats were of group II.

Fig. 2. Determinations on rats which fasted a total of 60 hours. Food was removed 36 hours before the first noon determinations. These rats were of group II.

Fig. 3. Determinations on the 5th and 6th days of the new feeding regimen. These animals were forced to feed between the hours of 9 a.m. and 5 p.m.

Fig. 4. Determinations on the 14th and 15th days of the new feeding regimen.

The figures in the right hand margin of each frame designate the number of the animal represented by the adjacent symbol.

subjected to experimental procedures for a longer period of time. One might expect that the diurnal rhythm in blood sugar concentration would be stronger and less variable in a group of young rats which had led an undisturbed existence prior to the fast than in an older group of rats which had been subjected to a number of previous bleedings and fasts. This is a possible explanation of the results obtained.

The data on animals 29 to 34 are graphically shown in figures 1 and 2, figure 1 being based on the data obtained during the forty-eight hour fast and figure 2 on the data obtained during the sixty-hour fast. We observe that in both cases the animals show greater variability than is shown by animals allowed to feed ad lib. However, in figure 1 the diurnal rhythm is still obvious in most most cases while in figure 2 it is obliterated in all animals except no. 30 and possibly no. 31.

In the last group of experiments rats were taught to change their usual feeding habits in an attempt to determine whether this would be accompanied by a similar change in their blood sugar cycle. Seven animals were allowed to feed only between the hours of 9 a.m. and 5 p.m. rather than during the hours of darkness. However, their period of maximum spontaneous activity still occurred during the night. This constituted a separation of the feeding and activity cycles. When these animals had completed adaptation to the new feeding regimen (a period of forty-eight to seventy-two hours as indicated by eventual maintenance of, or increase in, body weight) they were subjected to three sets of bleedings, the first beginning on the fifth day after initiation of the new feeding regimen, the second on the fourteenth day, and the third on the twentieth day.

The results of these experiments are summarized in part II of table 2. After five days on the new feeding regimen the blood sugar cycle was essentially unchanged (fig. 3). However, after fourteen days the blood sugar cycle was completely reversed (fig. 4), the noon values being approximately 10 per cent higher than the midnight ones, and the difference between the means being even more highly significant than that obtained on the fifth day. The results obtained on the twentieth day support those obtained on the fourteenth day. However, on the twentieth day the difference between the means is even greater. Obviously the normal blood sugar cycle persists for at least five days during enforced day feeding, but becomes completely reversed some time before the fourteenth day.

It is to be stressed that the typical spontaneous activity cycle of the animals persisted during these experiments. In most cases the total daily activity decreased 25 to 50 per cent. But its distribution over the twenty-four hours was essentially unchanged, the period of maximum activity still occurring during the hours of darkness. Hence the blood sugar cycle of the white rat accompanies the feeding cycle rather than the activity cycle.

Discussion. The foregoing data make it obvious that there is a diurnal rhythm in the blood sugar concentration of the normal albino rat allowed to feed ad lib. However, it is to be made clear that these data yield little information as to the exact nature of the diurnal curve. Midnight values may not represent the maxima or noon values the minima of the diurnal curve. But since Deuel and collaborators (3) found the digestive tract of the female rat to contain most food at 12 midnight, one at least has reason to believe that midnight determinations fall closer to the maximum point than to the point of inflection.

The observation of Cori and Cori (2) that the blood sugar concentration is an important factor determining the rate of liver glycogen formation would lead one to expect that the liver glycogen cycle would persist during fasting as long as the blood sugar cycle persists. This is apparently the case. Higgins, Berkson and Flock (7) found that the liver glycogen cycle of the white rat persists for one day after the removal of food but that during the second day it remains constant at a low level. This checks reasonably well with the present

experiments. On fasting rats, blood samples had to be taken within twenty-four to thirty-six hours after food removal (not counting the first six hours of a fast beginning at noon, for reasons explained above) in order to obtain a significant difference between day and night values.

Diurnal rhythmicity in the blood sugar concentration of an animal is most probably evoked by one or more of three principal factors, i.e., periodic changes in light (day and night), periodic habits in activity, and periodic habits in feeding. The first two of these factors are closely interrelated and were not altered at any point in these experiments. However, the third factor was isolated from the first two by the expedient of training the animals to feed during the daylight hours while the lighting conditions and spontaneous activity cycle were unaltered. Since it is possible completely to reverse the blood sugar cycle by such a procedure, one may say that cyclic feeding habits constitute the principal factor determining the diurnal rhythm in blood sugar of the rat.

The reversal of the blood sugar cycle obtained upon reversing feeding habits might have been predicted on the basis of the results of Higgins, Berkson and Flock (8). These workers found that by changing the hours of feeding they were able to shift the liver glycogen cycle of the white rat by six hours. It is of interest, however, that the reversal of the blood sugar cycle is not immediate but involves a "conditioning period" of more than five days. This is reminiscent of the seven to eight day conditioning period found by Hemmingsen and Krarup (6) to be necessary for a complete reversal of the activity cycle of the rat in response to a reversal of the periods of light and darkness. In each case the rhythm to be reversed is so completely integrated with the animal's general physiological "timing" that a reversal is obtained only gradually over a period of days even after the factor directly responsible for the cycle has been drastically altered.

It is noteworthy, finally, that the diurnal variations found to occur in the rat's blood sugar are not incompatible with findings on the human being. Man eats three meals a day and it is known (10) that his blood sugar shows three corresponding rises, one after each meal. The rat, by comparison, may be regarded as an animal which eats only one large meal a day (3), and consequently its blood sugar concentration shows one large increase each twenty-four hours.

SUMMARY

There is a diurnal rhythm in the blood sugar level of the normal, fed, female white rat. The difference between the noon and midnight values is statistically significant and is approximately 10 per cent. This blood sugar cycle persists during thirty-six to forty-eight hours of fasting but disappears during longer fasts.

By training animals to feed during the day it is possible to dissociate the feeding and activity cycles. In such a situation the blood sugar cycle is found to accompany the feeding rather than the activity cycle. However, a conditioning period of more than five and less than fourteen days is involved before the change in the blood sugar cycle is completed.

We may conclude that the diurnal cycle in the blood sugar of the white rat is due principally to cyclic feeding habits.

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A BLOOD VESSEL DEFECT IN SWINE SUFFERING FROM AN INHERITED BLEEDING DISEASE[1]

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Previous studies on an inherited bleeding disease in a strain of swine owned by the Missouri Agricultural Experiment Station show that the disease is transmitted by both sexes as a Mendelian recessive (1), and that the bleeders have a blood coagulation defect (2, 3).

We have shown more recently that the animals also have a very prolonged saline bleeding time (4), and an abnormally low capillary resistance (5). Neither of these symptoms can be readily attributed to the prolonged coagulation time (2, 3), and in this paper we wish to present evidence for a second abnormality, the improper functioning of injured blood vessels.

EXPERIMENTAL.[2] In studies on the prolonged saline bleeding time of bleeder swine (4), we often observed poor correlation between bleeding times and coagulation times. The coagulation times, obtained at the same time as the bleeding times (4), are shown for the first time in figure 1 of this paper. In all of the studies reported here, we used the following modification of the Lee-White (6) clotting time method: A vaseline-coated 19 gauge Wassermann needle is inserted into the marginal vein of the shaved ear, and 2 cc. portions of blood are collected successively in 4 paraffin-coated test tubes (inside diameter, $\frac{5}{8}$ inch, length, 6 inches). A blood flow of 4–8 cc. per minute is acceptable. The tubes are corked and placed in a bath at 37°. The first tube obtained is tilted at minute intervals until the blood is completely clotted. This procedure is repeated successively on the second, third and fourth tubes. The longest clotting time observed (usually tube 4) is recorded as the whole blood coagulation time. In figure 1, the determinations were carried out at approximately weekly intervals for a period of 3 months.

Figure 1 shows that the whole blood coagulation time is in the range of 36–104 minutes in the bleeders, and in the range of 19–53 minutes in the normal animals. These variations cannot be explained by differences in technique; therefore they must be due to actual variations in blood coagulability. There is, then, a natural 2 to 3-fold variation in the clotting time of both normal and bleeder animals, and some overlapping of clotting time values in the 2 groups. The average

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[2] We are indebted to Dr. Ralph Bogart, Department of Animal Husbandry, for maintaining the herd of bleeders from which our experimental animals were obtained.

clotting time (fig. 1) of the 3 bleeders for the 92-day experimental period is 67 minutes, and of the 5 normal animals, 31 minutes, a $2\frac{1}{6}$-fold difference. No differences between bleeder and normal were observed with respect to the quality and the firmness of the clotted blood, or its speed of retraction.

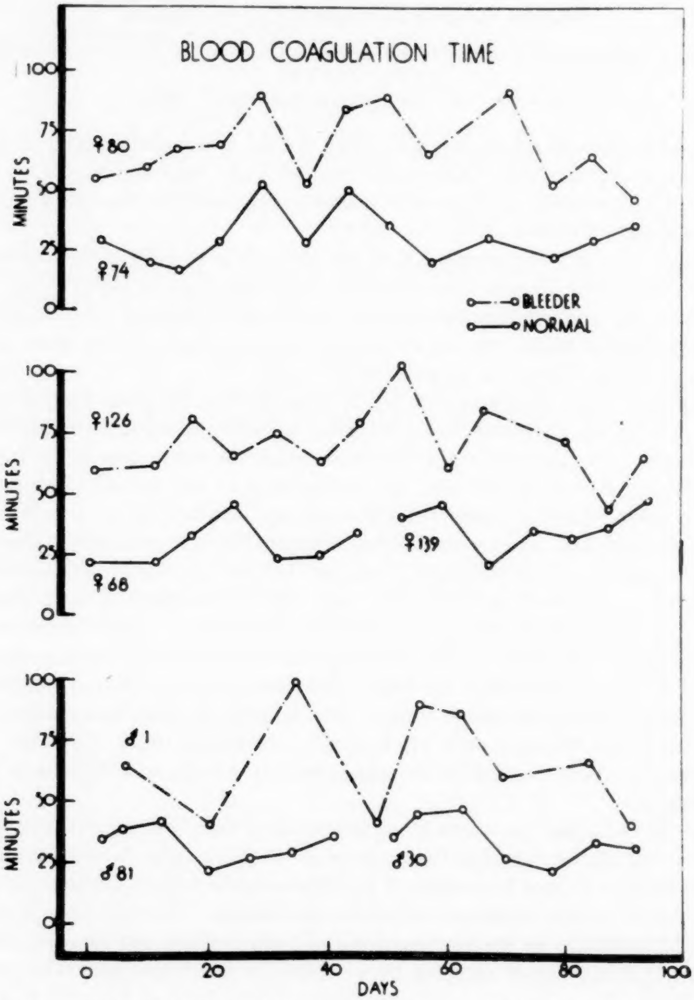

Fig. 1. A comparison of the whole blood coagulation times of bleeder and of normal swine

A comparison of coagulation times (fig. 1) with the saline bleeding times simultaneously obtained (see fig. 1, reference (4)), shows that in several cases (table 1) the coagulation time of the bleeders is short when the saline bleeding time is very prolonged, and vice versa. The anomaly of a short coagulation time

and a long bleeding time was also observed during the routine testing of a group of 4 month old bleeders (bottom of table 1). If the saline bleeding time were dependent on the whole blood coagulation time, one would expect a direct correlation, instead of the values shown in table 1.

We have been able to produce experimentally the combination of a prolonged saline bleeding time and a short coagulation time in bleeders. The data in table 2 show that small whole blood transfusions, while having no effect on the prolonged saline bleeding time, reduce the prolonged coagulation time to within the normal range for several hours.

Furthermore, we have been able to produce experimentally the reverse combination in normal animals, namely, a normal saline bleeding time in the presence of a very prolonged whole blood coagulation time. This is accomplished

TABLE 1

Simultaneous coagulation times and saline bleeding times in bleeders

ANIMAL	DAY (FIG. 1)	COAGULATION TIME	SALINE BLEEDING TIME
		min.	*sec.*
F80	92	48	600+
F126	87	44	550
M1	20	40	490
	91	36	600+
F80	71	90	360
F126	45	79	160
M1	34	99	260
	62	80	220
F7	—	39	600+
M34	—	45	600+
F6	—	42	600+

by administering heparin intravenously. Table 3 shows that the whole blood coagulation time of normal swine can be prolonged 2 to 20-fold by this treatment without affecting the short saline bleeding times of these animals. The coagulation values obtained in the normal swine are much longer than any observed to date in the bleeders (cf. fig. 1).

It is evident that the prolonged saline bleeding time of bleeders can be attributed neither to the prolonged coagulation time which exists simultaneously, nor to a defect in the quality or retraction properties of the bleeder clot. That it is an important symptom of the disease is shown by previous tests on 4–18 month old swine (4), and by the tests on one day old swine reported in table 4. On the basis of inheritance (1), the litter in table 4 should consist of 6 bleeders and 6 carriers, a ratio with which the bleeding time findings closely agree. It is apparent that a prolonged saline bleeding time exists in bleeders *even at birth.*

The relative independence of the saline bleeding time and the coagulation time in swine can be most readily explained by assuming that the 2 tests measure different phenomena. Since the saline bleeding time is not greatly changed by

fluctuations in the coagulability of the blood (tables 1, 2 and 3), it must be governed by changes outside of the blood, i.e., *changes in the blood vessel.* We therefore conclude that the saline bleeding time measures the *constricting ability* of

TABLE 2

The effect of blood transfusions on the saline and coagulation time of bleeders

ANIMAL	TREATMENT	BEFORE TREATMENT		AFTER TREATMENT		
		Saline time	Coagulation time	Hours after	Saline time	Coagulation time
		sec.	*min.*		*sec.*	*min.*
F10	2.2 cc. citrated blood* intrav. per kgm. body wt.	600+	89	2	600+	41
				4	600+	44
				6	600+	52
F6	Same as for F10	600+	88	3	600+	38
				5	600+	61

* An 18 gauge hypodermic needle was inserted into the marginal ear vein of a normal hog, and 4 parts of blood were allowed to flow from the needle into 1 part of 3.8 per cent sodium citrate solution.

TABLE 3

The effect of heparin on the coagulation time and saline bleeding time of normal swine

ANIMAL	TREATMENT	BEFORE TREATMENT		AFTER TREATMENT	
		Coagulation time	Bleeding time	Coagulation time	Bleeding time
		min.	*sec.*	*min.*	*sec.*
F213	0.5 mgm. Na heparin* intrav./kgm. body wt.	33	85; 85	660+ (5 min. after) 390 (19 min. after)	90 (7 min. after) 90 (17 min. after)
M159	0.3 mgm. Na heparin intrav./kgm. body wt.	33	65; 140	382 (5 min. after) 165 (17 min. after)	93 (7 min. after) 137 (14 min. after)
F76	0.2 mgm. Na heparin intrav./kgm. body wt.	53	125; 93	229 (5 min. after) 102 (23 min. after)	58 (14 min. after) 140 (17 min. after)

* One milligram Na heparin = 110 "Toronto units."

the blood vessels injured in the test, and that a prolonged saline bleeding time of the type found in bleeders is due to a *failure of the injured vessels to constrict normally.*

Evidence that an induced constriction of the blood vessels has a marked effect in shortening the prolonged saline bleeding time of bleeders is shown in table 5.

Small quantities of 1:1000 adrenaline (Parke, Davis) were injected with a 26 gauge needle into the tip of the shaved ear, and a lancet wound was made immediately about 5 mm. from the site of the injection. The wound was immersed in isotonic saline at 37° and the saline bleeding time was determined in the usual manner (4). Chester White-Poland China crosses, and purebred Poland Chinas were tested. The 3 day old animals shown in table 5 are from the same litter (table 4).

TABLE 4

Saline bleeding time tests on a litter of Chester White-Poland China swine one day of age

BLEEDERS	SALINE TIME	CARRIERS	SALINE TIME
	sec.		*sec.*
M36	600+	M34	60
M30	600+	M31	75
F30	600+	F31	85
F33	600+	F35	65
M35	600+	F34	55
F37	460		
F36	400		

TABLE 5

The effect of adrenaline on the prolonged saline bleeding time of bleeders

ANIMAL	F1	F9	M5	F30	M30	F33	F36	M35	M4	F10	F7
Skin color (W-white; B-black)	W	W	W	W	W	B	B	B	B	B	B
Breed (C-cross; P-pure)	C	C	C	C	C	C	C	C	C	P	P
Age (days)	156	200	40	3	3	3	3	3	38	463	463
Cc. adrenaline (1:1000) injected	0.05	0.05	0.05	0.05	0.02	0.05	0.05	0.05	0.05	0.05	0.05
Saline time before injection (sec.)	600+	600+	260	600+	270	375	360	600+	600+	420+	600+
Saline time after injection (sec.)	98	20	60	115	107	560	330	480	600+	600+	600+

It is evident from table 5 that Chester White-Poland China bleeders with white skins show a reduction of the prolonged saline bleeding time to normal after the injection of adrenaline. Because of this hemostatic effect, adrenaline was found to be very useful in the treatment of surface hemorrhages in these animals. Thus, a tooth socket hemorrhage in F1 (table 5) was stopped at once by the injection of 0.1 cc. of 1:1000 adrenaline into the gum adjacent to the bleeding site. White F9 incurred a deep cut on the edge of the snout which bled for more than 2 hours. The injection of 0.05 cc. of 1:1000 adrenaline on each side of the cut

stopped the bleeding immediately; there was no recurrence of the hemorrhage in spite of pronounced vessel dilatation 20 minutes later. This same animal bled for more than 5 hours from a deep cut in the floor of its mouth. Injection of 0.05 cc. of 1:1000 adrenaline on each side of the cut also stopped this hemorrhage in a few minutes, with no recurrence during subsequent dilatation of the affected vessels.

We are at present unable to explain why injected adrenaline has no effect (table 5) on the prolonged saline bleeding time of black-skinned Chester White-Poland China bleeders, and black-skinned purebred Poland China bleeders. In addition, injected adrenaline has no effect in controlling hemorrhage in these animals. The cause of this anomaly may lie in hereditary factors, i.e., a dominance of the black Poland China bleeder strain in the black bleeders, and a dominance of the Chester White normal strain in the white bleeders.

Discussion. The data presented above are in good agreement with the "two-stage" hemostasis theory recently proposed by Macfarlane (7). According to Macfarlane, the first stage of hemostasis in the smaller blood vessels of mammals consists of a temporary constriction of the wall of the severed or otherwise injured vessel; this slows down or stops the loss of blood. The second stage consists of a firm coagulation of the blood and a retraction of the blood clot in the lumen near the temporarily constricted part of the injured vessel. Following this second stage, dilatation of the vessel occurs, but the plugged condition of the injured area prevents further loss of blood. Our findings indicate that both the first and second stages of hemostasis are abnormal in the bleeder swine. Furthermore, our tests with adrenaline show that the type of vessel defect is not exactly the same in the white-skinned and the black-skinned bleeders.

SUMMARY

Evidence is presented to show that swine suffering from an inherited bleeding disease have a blood vessel defect in addition to the previously observed coagulation defect.

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CIRCULATORY REACTIONS OF RATS TRAUMATIZED IN THE NOBLE-COLLIP DRUM

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These investigations were undertaken during the summer of 1941 and at intervals through the following year to obtain data supplementary to those made by Noble and Collip. Some of the experiments are confirmatory of those published by Noble and Collip (1, 2). In addition, a detailed study was made of blood pressure changes, hematocrit, and plasma volumes, also observations on the visceral and peripheral capillary circulation.

In the course of the observations, it was soon realized that the trauma induced by drumming was very general and caused much congestion, especially in the gut. This made it difficult to differentiate between direct and indirect effects of the trauma on the capillary circulation. Hence, the observations described in this paper on the capillaries are not to be considered as strictly referable to the effects of traumatic shock on capillaries remote from the trauma.

METHODS. Rats, with their paws taped to render them helpless, were placed in a drum, 16 inches in diameter and 7 inches deep, and the drum rotated at a speed of 45–50 revolutions per minute. Two 2-inch shelves, fastened on opposite sides on the inner circumference of the drum, carried the rat with each revolution part way up the circumference until it slipped off and fell. This procedure caused the rat to sustain at least one and more often two falls per revolution thereby undergoing cumulative trauma with the successive falls. It may be stated that the rats gave no sign of pain. They were evidently thrown into a semiconscious state at the first fall, and the successive falls were sufficiently rapid in sequence to prevent recovery until they were removed from the drum.

The experimental data of Noble and Collip show that rats, weighing about 150 grams and subjected to 800 revolutions, underwent injuries with a 100 per cent mortality, while of those subjected to 300 turns the mortality was 8 per cent. In the intermediate range of 600 revolutions the mortality was approximately 75 per cent. The period of survival of the fatal cases, subjected to this intermediate range of trauma, varied from 20 minutes to 10 hours, the great majority (ca 70 per cent) dying within 45 to 90 minutes. These results, indicating the statistically consistent type of trauma involved, have been confirmed by us except for a slightly longer survival period in the 650 revolution range (60–120 min.) in experiments on over 800 rats.

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About 500 rats were subjected to 650 revolutions of the drum. A certain percentage of these (10–15 per cent) either died in the drum, exhibited gross hemorrhage (usually in the gut), sustained intracranial hemorrhages and fractured skulls, or died within 20 minutes after removal from the drum. These were discarded from consideration. The remainder survived for at least one hour or two after removal from the drum.

Blood pressures were determined repeatedly on the same rat. The method, developed in this laboratory, is described by Duncan, Hyman and E. L. Chambers (3). It is based on the microscopic observation of arteriolar blood-flow in the interdigital web of the hind foot while the femoral artery is being occluded by a pneumatic cuff around the upper part of the thigh. The pressure of the normal, unanesthetized rat showed variations in different rats from 90 mm. to 140 mm. Hg, the pressure of the majority lying between 110 mm. and 120 mm. The accuracy of the method may be indicated by the fact that successive readings on the same rat by different observers were reproduced within differences of 2–4 mm. Hg. The readings were taken at the moment a flow was detected when the pressure was released on the femoral artery. They may be regarded as the systolic pressures. The advantage of the method is in the absence of any surgery in the procedure.

The hematocrit volumes were obtained by the Van Allen method and the plasma volumes by the dye method introduced by Keith, Rowntree and Geraghty.

The capillary circulation was observed simultaneously in the subcutaneous tissue of the skin between the toes of the hind leg and in the meso-appendix, a triangular flap of mesentery between cecum and ileum. In order to have the rat sufficiently anesthetized to permit exposure of the meso-appendix after the trauma, it was given a subcutaneous injection of sodium pentobarbital solution (0.45 cc. of 1 per cent solution per 100 grams body weight) immediately before placing it in the drum. The anesthetic then became effective at about the time the rat was removed from the drum. Since the trauma is one of direct injury to the viscera it was found necessary to offer some degree of protection to the region selected for observation in order to avoid local petechiae. This was accomplished by strapping the lower abdomen with a strip of 1 inch adhesive with cotton interposed directly over the cecum. The protection afforded was not sufficient to prevent the development of shock symptoms. The cecum of these rats was hyperemic but showed no bruises or local hemorrhages such as usually occur when the lower abdomen is unprotected. After exposing the cecum the meso-appendix was spread over a horse-shoe shaped ribbon of lucite. The viscus was covered with cotton and the preparation kept moist by a drip of Ringer Locke solution buffered to pH 7.4 and thermostatically controlled at 38°C.

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RESULTS. 1. *Evidence for the site of action of the trauma.* The rats in the

rotating drum suffered minor contusions over the bony prominences, principally the dorsal margin of the ilium, the scapula, the base of the skull, and the snout. The teeth were frequently broken, and the scrotal sac contused.

Experiments were made to determine which regions of the body had to be traumatized to induce fatal shock. The rats were protected over various regions with pads of absorbent cotton which were held in position with adhesive tape. It was found that padding over the head, shoulder, back and scrotum did not protect the animal provided the abdomen was left exposed. These rats went into fatal shock with the same number of revolutions as those left completely unprotected. When the abdomen was well-padded, the rat withstood 1000 revolutions without developing fatal shock.

In rats with the entire abdomen protected the blood pressure dropped to about 50 mm. Hg immediately after removal from the drum. In the great majority of cases the pressure then rapidly rose to normal values. Autopsies on these animals, killed at varying intervals after the trauma, revealed no gross visceral congestion.

The conclusion is that the action of the Noble-Collip drum, within the range of 650 revolutions, induces a fatal, shock-like state largely through direct trauma to the viscera.

2. *Signs and symptoms of the shocked state in unprotected rats. External signs.* These were the same as described by Noble and Collip (l. c.). Immediately on removal from the drum the rat was comatose. The ears, tail and feet showed an intense pallor and were cooler than normal. During the following 15–20 minutes the respiration which was at first shallow and rapid, became slower and more normal. The initial pallor decreased, and the rats became more responsive to cutaneous stimuli. This improvement persisted for a variable time, after which, in fatal cases, the animals became progressively less responsive until death.

Internal signs. The gastro-intestinal tract showed marked vascular engorgement chiefly in the duodenum, upper jejunum, cecum and, to a lesser extent, the lower ileum. Varying amounts of free fluid were usually present in the lumen of the gut, especially in the duodenum and upper jejunum. The mucosa frequently showed marked vascular engorgement, though no definite ulcerations were ever observed. The mesenteric lymphatics and the thoracic duct were occasionally engorged and, sometimes, the contained fluid was sanguinous. The liver, spleen, kidneys, and adrenals gave evidence of vascular engorgement, likewise the lungs which frequently contained petechiae. Gross examination of the brain revealed no pathology except in rats discarded because of cranial hemorrhage and fracture. The urine was scanty, concentrated, dark, and, in rare instances, grossly bloody.

Rectal temperature. There was a consistent drop in temperature from the normal of 98°–100° to 93°–95°F. immediately following drumming. It persisted below normal in all fatal cases.

Blood pressure. In some rats blood pressure readings were unobtainable because of complete circulatory stasis in the skin vessels. In about 250 cases only a few blood pressure readings were taken. In 50 cases readings were taken at 10 minute intervals on each rat over a period of 2–4 hours. Representative fatal

cases showed a primary fall to 30–40 mm. Hg, followed, within 10–20 minutes, by a temporary rise to 60–70 mm. Hg; after which the pressure gradually fell during a period of 2–4 hours until death ensued. A number of cases in this group maintained extremely low pressures with no rise throughout until death.

Blood pressure curves of the rats which survived also showed an initial drop which persisted for about 10–20 minutes and then rose to the normal level within 1 to 20 hours.

Hematocrit. Blood samples were withdrawn from the jugular vein at 15–20 minute intervals after removal from the drum. Because of the small total blood volume of the rat, care was taken to remove minimal amounts, on the average, not over 0.3–0.5 cc. Hematocrit volumes were determined in duplicate in about 50 rats using the Van Allen method.

Hemoconcentration was consistently obtained. In those which recovered, the hematocrit values never rose more than 6–8 per cent above normal, and normal levels were reached within 1–3 hours after the trauma. In those which eventually died (1–12 hours after the trauma) the hematocrit values began to rise 30–60 minutes after removal from the drum. Levels of 9–20 per cent above normal, with an average of 12 per cent, were reached within 1–3 hours, which persisted until death. These results are in accord with the hemoglobin determinations reported by Noble and Collip (l. c.).

Plasma volumes. Plasma volumes were determined by the T-1824 dye method. The procedure employed was essentially that described by Beckwith and Chanutin (4). The dye determinations were taken on samples from the right atrium. Experience of other investigators with shocked dogs has indicated that samples from veins gave variable results.

All the rats were subjected to drum trauma within 24 hours after the control determination. In all the cases in which there was no gross evidence of hemorrhage or fluid loss into the gut, the trauma was found to induce a decrease in total plasma volume of about 12 to 15 per cent. A maximum value of 20 per cent was obtained in cases in which gross hemorrhage was visible in the duodenum. Parallel hematocrit readings were not done.

3. *Diffusion of the dye, T-1824, from blood vessels.* An indication of the site of fluid loss was obtained by observing the escape of the dye, T-1824, intravenously injected prior to trauma. Gross examination showed that the skeletal muscles were diffusely bluer than in non-traumatized rats. There was also evidence of considerable loss of the dye into the gastro-intestinal tract, over bony prominences and, in females, into the subserosal regions of the uterus and its cornu.

The change affected by the drum trauma to diffusion of the dye T-1824 was also tested by the local application of heat before and after the trauma. Tests were first made on normal rats by injecting T-1824 (0.2 to 0.3 cc. of a 1 per cent solution in mammalian Ringer) into the jugular vein. Three regions of the skin on one side of the abdomen were then exposed to heat for 10, 20 and 30 seconds, respectively, by applying glass tubes through which hot water was flowing, the temperature at the outflow being kept at 46°C. No bluing effect was noted in any of the heated regions. The same rat was then subjected to 600

revolutions. Upon removal from the drum, the regions of the abdomen previously exposed to heat still showed no color change. The other side of the abdomen was then treated with the same heat-application technique. The region exposed for 20 seconds was slightly blue while that exposed for 30 seconds was a deeper blue.

4. *Visceral and cutaneous capillary circulation.* The following data represent observations on tissues in which the effects of direct trauma tended to be superimposed on the more generalized shock condition. The description of the observed changes in the capillary circulation was taken from 20 rats.

a. The effect of the trauma on the circulation was studied by exposing the meso-appendix of rats at varying intervals after they had been drummed for 650 revolutions. Two of the criteria, that were especially noted, were the sensitivity of the muscular vessels of the capillary bed to epinephrine, and their vasomotion. This vasomotion is independent of the arterial pulsations and consists of a widening and a narrowing in slow, irregularly periodic sequences, varying from about 15 seconds to 3 minutes (5).

Five minutes after removal from the drum the capillary circulation was hyperemic and slow. The terminal arterioles were partially dilated and lacked the vasomotion characteristic of arterioles in normal rats. However, there was a pulsatile flow evidently transferred from the arteries. When first observed, the circulation in the larger vessels was slow but it gradually became accelerated, a condition which was found to correspond with the appearance of external signs of improvement noted in unanesthetized rats. In some cases a reversal of flow occurred in the smaller veins, the blood backing into the capillaries. The veins and arteries were hypersensitive to epinephrine. A concentration of 1 part in 2 million, which in the control, nembutalized rat induces slight arteriolar constriction, completely occluded the terminal arterioles and induced contractions of the veins followed by uneven relaxations and irregular constrictions which lasted 20–30 minutes.

Thirty minutes after removal from the drum, hemoconcentration was indicated by the closer packing than normally of the blood corpuscles in the capillaries while the flow was appreciably slowed in the arteries and veins and sluggish in the capillaries. The indications were that the hemoconcentration either had occurred previous to the period of observation or was occurring remote from the area under observation. The terminal arterioles and precapillaries still showed no vasomotion and now were hyposensitive to epinephrine, constricting only with concentrations of 1 part in 1 million or higher.

The application of heat, by causing the drip of Ringer's solution to rise to 40°C, resulted in the development of hemorrhages throughout the bed, indicating an abnormal fragility of the capillary walls. In the normal rat this amount of heat produced only a hyperemic flow with no sign of local hemorrhage.

Sixty or more minutes after removal from the drum the capillary bed was relatively ischemic with very little flow. Most of the blood coming from the arteries by-passed the capillary bed by flowing directly through cross shunts to the corresponding veins. The arteries were considerably constricted. The smaller venules contained a sluggish and resurgent flow with a tendency of the

blood cells to be stagnated. Considerable back-flow from the veins into the capillary bed was observed, causing further stagnation.

About 10–15 minutes before death, a sticking of leucocytes became evident in the walls of the smaller veins and venules. Responses of the muscular vessels to epinephrine were extremely poor, the application of a concentration of 1 part in 1 million yielding little or no response.

b. Peripheral circulation in the interdigital web and scrotum. Immediately on removal from the drum the blood in the capillary beds was found to be stagnant, the blood cells in the capillaries merely oscillating to and fro. There was no evidence, however, of true stasis in which the red cells become closely packed through the loss of plasma from the vessel. In some of the more deeply placed arterioles a slow, continuous flow usually persisted until shortly before death. The superficial venules were narrowed and appeared abnormally pale, containing only a very few red cells. No recovery of flow occurred in those rats which died within 2–3 hours after the trauma.

SUMMARY

1. *Blood pressure.* Exposure of 150 gram rats to 600–650 revolutions in the Noble-Collip drum resulted in a lowered blood pressure which, in fatal cases, persisted below 60–70 mm.

2. *Hemoconcentration and plasma volume.* *a.* The hematocrit values always showed an increase, averaging 12–15 per cent above the initial normal values. *b.* The total plasma volume in the majority of cases, showed a decrease of about 12–15 per cent.

3. *Site of trauma.* Protection of the abdomen during the drumming prevented the development of a shock-like state in rats subjected to drumming up to 1000 revolutions.

4. *Capillary circulation in the rats subjected to 650 revolutions of the drum.* *a. Visceral.* i. The hyperemic state of the capillary circulation in the mesentery was probably due, in large part, to the direct trauma on the viscera. A slowing of the flow and absence of arteriolar vasomotion progressively became apparent concurrently with the fall in blood pressure. ii. The sensitivity to epinephrine was at first increased, particularly of the veins. It then progressively decreased to considerably below the normal response. iii. Hemoconcentration became visibly apparent within 30 minutes after the trauma. iv. In the terminal stages, the flow became increasingly restricted and was accompanied by a progressive stagnation, especially in the venous portion of the capillary bed. *b. Peripheral.* From the time of removal from the drum until death, the circulation was found to be stagnant, with a slow flow only in a relatively few vessels. There was no sign of local hemoconcentration.

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PLASMA COAGULATION AND FIBRINOGENOLYSIS BY PROSTATIC FLUID AND TRYPSIN[1]

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Both crude and crystalline trypsin are known to clot oxalated blood (1–3). Eagle and Harris (3) discovered that trypsin does not clot fibrinogen and that in appropriate amounts trypsin digests fibrinogen and fibrin, and clots oxalated plasma; trypsin also digests thrombin, and prothrombin (4). Ferguson and Erickson (5) found that crystalline trypsin, 1–2 mgm. acting alone were optimal for clotting citrated plasma, but when potentiated by calcium ions smaller amounts yielded quick solid clots.

Huggins and Neal (6) observed that the prostatic secretion of man and dog normally contains proteolytic agents capable of digesting fibrinogen and fibrin of certain species. The agent which disolves fibrin closely resembles the fibrinolysin obtained as a metabolic product of hemolytic streptococci by Tillett and Garner (7). Evidence was presented that this prostatic fibrinolysin was different from the activity which destroyed fibrinogen, fibrinogenase. Both proteolytic agents are present in human and dog prostatic fluids but in different amounts; human prostatic secretion contains much more fibrinolysin than fibrinogenase and canine prostatic fluid the reverse. Moreover, trypsin as recognized by the splitting of denatured hemoglobin according to the method of Anson (8) is constantly present in dog prostatic fluid and less often in human secretion.

In this paper we report the coagulation of oxalated plasma by prostatic fluid of the dog (DPF) and compare the clotting and fibrinolytic properties of prostatic fluid and trypsin.

METHODS AND MATERIALS. Prostatic fluid from 15 dogs was obtained by pilocarpine stimulation following the prostatic isolation procedure of Huggins, Masina, Eichelberger and Wharton (9); this fluid was sterilized by passing it through a Seitz filter. Human prostatic fluid from eight normal men was procured by massage of the prostate, *per rectum*, and was tested immediately after centrifugation.

Oxalated plasma was used throughout. Potassium oxalate, 2–8 mgm. per 1 cc. of blood was used as anticoagulant. Rabbit blood was obtained by cardiac puncture in a syringe containing the oxalate dissolved in saline. Certain specified samples were recalcified with calcium chloride, 1.5 per cent, 0.33 cc. The commercial trypsin used, "Trypsin 1:250", was manufactured by the Difco Laboratories. It was dissolved in saline and filtered; the pH of the solution, determined electrometrically, was 6.5. Heparin employed was that of Hynson, Westcott and Dunning and was freshly dissolved in saline before using; the manu-

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facturers stated that 1 mgm. of this preparation prevented the coagulation of 7.5 cc. of cat's blood for 24 hours. Trypsin content of prostatic fluid was determined by the method of Anson (8) and the results are expressed in the units of Kolm, Shay and Gershon-Cohen (10).

Beef plasma was freed of prothrombin by adsorption with magnesium hydroxide and fibrinogen was prepared by the method of Smith, Warner and Brinkhous (11).

In all of the experiments, plasma 1 cc., was mixed with 0.5 cc. of fluid to be tested, either undiluted or diluted with saline and immediately placed in a water bath at 37°C.; the times of coagulation and of lysis were determined. Unless otherwise stated, *all of the data in this paper refer to the effect on plasma, 1 cc.*

RESULTS. *Coagulant action of prostatic fluid.* DPF (from 15 dogs) always induced firm coagulation of oxalated rabbit plasma without added calcium. The calcium content of DPF is low, 0.3 mM per kgm. of water (9). The time of clotting induced by DPF varied from 4 to 31 minutes, the usual time being about 10 minutes. With increasing dilution of DPF the clotting time became progressively increased. In a typical experiment 1 cc. of oxalated plasma was clotted by 0.5 cc. of DPF in 9 minutes; by 0.1 cc. in 20 minutes; by 0.05 cc. in 35 minutes; and by 0.006 cc. in 5 hours. With 9 specimens of oxalated beef plasma, DPF induced clotting in 22–50 minutes, without added calcium. In two instances diluted DPF, 0.006–0.05 cc. induced clotting of dog plasma, while in stronger concentrations no clotting occurred and the fibrinogen was destroyed. DPF never induced clotting of human blood in any concentration in 12 tests.

The prostatic fluid of 8 men always failed to clot oxalated rabbit plasma. The addition of calcium chloride after 4–21 hours resulted in a prompt, firm clot.

Effect of excess oxalate and of heparin on coagulation of rabbit plasma by DPF. In drawing rabbit blood, precautions were taken to minimize spontaneous thrombin formation. In addition to drawing the blood in a syringe containing liquid oxalate, the first 10 cc. of blood flowing from the heart were discarded and with the needle still in place a second sample was drawn into a fresh syringe. DPF consistently induced clotting of this oxalated plasma. An excess of potassium oxalate, 16 mgm. per 1 cc. of blood, still permitted this firm coagulation.

The addition of heparin, 3 mgm. to 10 mgm. per 1 cc. of blood modified the coagulation of oxalated rabbit plasma by DPF in two respects. Instead of a firm clot, a heavy flocculation occurred in the plasma, which remained liquid. On microscopic examination, the coagulum consisted of interlaced fibrils resembling fibrin. No precipitation occurred in the plasma, from which the clot had been removed by centrifugation, on heating to 56° or on half saturation with NaCl. This flocculation occurred at a slower rate than did the coagulation of plasma by DPF without heparin; the flocculation developed in 21 minutes to 2 hours, as compared with its coagulation in ten minutes without heparin.

Effect of heating on dialysis of DPF. Storing DPF in a refrigerator at 4°C. did not result in a loss of thrombin or fibrinolytic activity. The dialysis of DPF in a cellulose bag against running tap water at 4°C. for 5 days had no effect on its ability to clot oxalated dog or rabbit plasma or to effect subsequent fibrinolysis.

Ashing DPF at 550°C. with resolution of the ash in distilled water equal to the original amount abolished its coagulant and lytic actions.

Heating DPF at 60°C. for 30 minutes still permitted it to coagulate rabbit plasma in the same time as occurred with unheated DPF; heating to 70°C. for 10 minutes abolished the clotting action of DPF. Exactly similar findings were observed with heated trypsin solutions in saline.

Coagulative capacity of DPF on prothrombin and fibrinogen. DPF always failed to clot purified beef fibrinogen solutions. When prothrombin was added to a mixture of DPF and fibrinogen clotting occurred in 6 minutes. Clotting was not observed when DPF was added to beef plasma from which prothrombin had been adsorbed with colloidal Mg $(OH)_2$ after adjusting the adsorbed plasma

TABLE 1

The effect of dog prostatic fluid on coagulation and on lysis of fibrinogen and fibrin of plasma

One cubic centimeter of oxalated plasma + 0.5 cc. of prostatic fluid, undiluted or diluted to 0.5 cc. In series A, $CaCl_2$ was added immediately after mixing; in series B, $CaCl_2$ was added 6 hours after incubation.

	AMOUNT OF PROSTATIC SECRETION IN TEST (CC.)							
	0.5	0.1	0.05	0.025	0.016	0.01	0.006	0.005
Human plasma: Series A, calcium added immediately								
Coagulation (minutes)...	none	4	4	4	4	4	4	4
Fibrinolysis (hours)....		7	18	none	none	none	none	none
Human plasma: Series B, no added calcium								
Coagulation (minutes)...	none	none	none	none	none	none	30*	30*
Fibrinolysis (hours)......							none	none
Rabbit plasma: no added calcium								
Coagulation (minutes)...	10	30	30	180	180	5*	5*	5*
Fibrinolysis (hours)......	16	16	16	none	none	none	none	none

* No clotting in 6 hours: calcium then added.

to pH 7.4. DPF added to phosphated plasma produced clotting in 20 minutes. It was assumed that the magnesium solution had more effectively removed prothrombin than did calcium phosphate.

Trypsin content of DPF. The tryptic activity of 12 fresh specimens of DPF on denatured hemoglobin was determined (8) and values were obtained ranging between 0.1 to 1.1 unit, an average of 0.59 unit; 1 trypsin unit represents such tryptic activity as develops chromogen equivalent to tyrosine, 1 mgm., in 1 minute under the stated conditions, expressed per 100 cc. (10).

Action of prostatic fluid and trypsin on human and rabbit plasma. DPF added to oxalated human plasma never induced coagulation but destroyed fibrinogen in concentrations as low as 0.01 cc. (table 1). Prompt clotting occurred in the unclotted specimens when beef fibrinogen was added after incubation for 6

hours. Added to oxalated rabbit plasma, coagulation occurred with as little DPF as 0.016 cc.

Trypsin in 10 mgm. amounts destroyed human and rabbit plasma fibrinogen before clotting occurred. Trypsin in amounts 1 mgm. to 5 mgm. caused clotting of human plasma, but smaller quantities were ineffective (table 2). Rabbit plasma was clotted with much smaller amounts of trypsin than human plasma; trypsin in amounts of 0.1 to 5 mgm. being effective. Human fibrin was digested with slightly less amount of trypsin than rabbit fibrin.

Heparin was found to interfere with the digestive action of trypsin on fibrinogen. In the presence of heparin 3 mgm., trypsin 10 mgm. produced flocculation of dog plasma, an amount which promptly destroyed fibrinogen in the absence of heparin.

TABLE 2

Coagulation and fibrinolysis induced by trypsin in human and rabbit plasmas

One cubic centimeter of oxalated plasma + 0.5 cc. trypsin solution

	AMOUNT OF PANCREATIC TRYPSIN IN TEST (MGM.)								
	10	5	3	2.5	2.0	1.5	1.0	0.5	0.1
Human plasma									
Coagulation (minutes)	none	15	15	15	15	40	18 hr.	none	none
Fibrinolysis (hours)		$\frac{1}{4}$	5	18	18	none	none	none	none
Rabbit plasma									
Coagulation (minutes)	none	3	15	15	15	15	15	90	18 hr.
Fibrinolysis (hours)		$\frac{1}{4}$	5	18	none	none	none	none	none

DISCUSSION. Pancreatic trypsin according to its concentration affects plasma in various ways. Large amounts destroy fibrinogen, smaller quantities coagulate oxalated plasma and subsequently the fibrin dissolves, while still smaller amounts clot plasma without destroying fibrin (3). Ferguson (15) has concluded that both crude trypsin and pure crystalline trypsin show parallelism of behavior with respect to fibrinogenolysis and fibrinolysis.

Human prostatic secretion has a powerful fibrinolytic action but destroys fibrinogen at a slow rate (6). This fluid never clots oxalated rabbit plasma and inconstantly contains trypsin according to the method of Anson. This is further evidence that the principal proteolytic enzyme of human prostatic fluid is fibrinolysin similar to the fibrinolysin obtained from hemolytic streptococci by Tillett and Garner (7) and that it differs in its activity from the main proteolytic enzyme of prostatic fluid of the dog and from pancreatic trypsin.

Many of the properties of DPF resemble trypsin, which is constantly detectable in small amounts in this fluid. Both DPF and trypsin coagulate oxalated rabbit plasma but not fibrinogen or plasma from which prothrombin has been adsorbed. Both clot rabbit plasma in concentrations too low to cause fibrinolysis. Both the commercial trypsin used and DPF were active after heating at 60°C. for 30 minutes and both were destroyed at 70°C. Heparin added to oxalated rabbit plasma did not prevent coagulation by trypsin or DPF.

Ferguson and Glazko (12) found that heparin decreased the proteolytic activity of trypsin and in line with this observation we found that heparin, 3 mgm. permitted trypsin 10 mgm. to clot oxalated plasma, an amount which destroyed fibrinogen before clotting in the absence of heparin.

There are also points of similarity between the proteolytic activity of DPF, trypsin and the enzyme prepared from chloroform treated plasma by Tagnon and associates (13, 14). Tagnon's preparation from dog plasma (13) clotted plasma containing prothrombin, but did not coagulate fibrinogen. At low concentration, the chloroform plasma globulin from human blood (14) clotted fibrinogen while at higher concentration coagulation was followed by fibrinolysis and in still larger amounts fibrinogenolysis resulted with no coagulation.

One important difference was observed between the proteolytic effects of trypsin and the chloroform plasma globulin on the one hand and DPF, in that DPF has far greater activity in destroying human plasma fibrinogen than in destroying fibrin; with diluted amounts of DPF, fibrinogen is digested before clotting occurs while the same amounts did not cause fibrinolysis of blood which had been recalcified. Trypsin in large amounts destroys fibrinogen promptly while smaller amounts are ineffective. The smallest amounts of DPF clotting rabbit plasma destroyed human fibrinogen in 5 hours while the smallest amounts of trypsin effecting clotting did not digest this protein (tables 1, 2).

It should be stressed that the enzymes of prostatic fluid have not been isolated in a chemically pure state and that deductions at the present time are based on the physiological activity of these fluids. Much evidence has shown the proteases are to be considered as "enzyme and inhibitor" systems: until the conditions and degree of activation of these modalities are more clearly defined than at present conclusions drawn as to the chemical nature of the prostatic proteases must be tentative. However, it is concluded that the proteolytic properties of DPF are functionally not identical with pancreatic trypsin, although the similarity of many properties suggests that fibrinogenase and trypsin are closely related.

SUMMARY

The prostatic secretion of normal dogs in effective amounts constantly clots oxalated rabbit and beef plasmas, and a mixture of fibrinogen and prothrombin, but does not coagulate fibrinogen alone or prothrombin-free plasma. This clot formation proceeds in the presence of large amounts of oxalate and heparin, although with heparin a flocculated precipitate develops instead of a firm clot. Dog prostatic fluid does not coagulate human plasma since fibrinogen is destroyed before clotting occurs. Thrombic activity of dog prostatic fluid is stable for months at 4°C. It does not disappear on prolonged dialysis against tap water or on heating at 60°C. for thirty minutes but is destroyed at 70°C.

Human prostatic fluid does not coagulate oxalated plasma.

Many proteolytic properties of dog prostatic fluid resemble those of pancreatic trypsin; an important difference is the greater activity of prostatic secretion even

in low dilution in destroying plasma fibrinogen compared with fibrin. Trypsin does not possess this effect.

The principal proteolytic activity of dog prostatic fluid, *fibrinogenase*, resembles, but is not physiologically identical with, trypsin; the chief proteolytic enzyme of human prostatic fluid is *fibrinolysin*.

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COPPER-INDUCED PSEUDOPREGNANCY FACILITATED BY PRETREATMENT WITH ESTROGEN[1]

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In a previous report (1) it was shown that pseudopregnancy can be induced in the rat by an intravenous injection of a copper solution at the time of estrus. Furthermore it was shown that the estrous rhythm of the rat is not appreciably altered by similar injections during the metestrous or diestrous phases of the cycle. These results suggested the possibility that the responsiveness of the rat varies with the cyclic fluctuations of endogenous estrogen. If this is the case, pretreatment with estrogen should make it possible to induce pseudopregnancy by injections of copper solution at other stages of the estrous cycle. The experiments described in this paper were designed to test this possibility.

Materials and methods. The rats used in this study were of the Wistar strain, bred and raised in our stock colony. Six to 8 rats were kept in a cage and the experimental observations were started after they were 80 to 150 days of age. Individual daily vaginal smear records were kept throughout the investigation until the animals were sacrificed. As a rule the smears were taken at about 9 o'clock a.m. each day and the rats were used only after they had exhibited two or more normal estrous cycles. After an injection, a prolonged diestrus (10 to 14 days) was considered evidence of an induced pseudopregnancy. The technique of the intravenous injection and the preparation of the copper acetate solution was the same as described previously (1).

The estrogen used in these experiments was crystalline estrone[3] dissolved in sesame oil. In order to avoid the toxic effects as described by Crafts (2) the oil was treated by the method of Bruce and Tobin (3) and the dose kept very small. In our experience, volumes of oil greater than 0.1 cc. are not absorbed from subcutaneous sites within a 24 hour period. By the use of a special 0.25 cc. Luer syringe it was possible to inject doses ranging from 0.01 cc. to 0.05 cc. in volume. The concentration of the estrone in the oil was 400 gamma per cubic centimeter.

Experimental. Fifty rats were given an intravenous injection of 1 per cent copper acetate solution (0.1 cc.) at the time of estrus with a resultant pseudopregnancy in every case. Seventeen rats were injected with copper at the time of metestrus and 17 at the time of diestrus but pseudopregnancy did not occur in any case. However, when estrone (8 to 12 gamma) was given during the

[1] This research was supported by an appropriation from Bankhead-Jones funds (Bankhead-Jones Act of June 29, 1935).

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[3] The estrone was generously supplied by Dr. Max Gilbert of the Schering Corporation.

afternoon of the day of an estrous smear and then the copper was given the next morning (normally metestrus) there was a resulting pseudopregnancy in every case (37 rats shown in table 1). Similarly when estrone (8 to 12 gamma) was given on the day of metestrus and then the copper given on the next day (normally diestrus) pseudopregnancy was induced in a majority of the rats.

Varying the dose of estrone makes it apparent that a dose of 4 gamma is effective when given at the time of estrus but not when given at metestrus. Similarly, doses of 8 to 12 gamma of estrone are not uniformly effective when given at metestrus. The doses of estrone used in this study did not appreciably alter the rhythm of the vaginal smears except that estrone given at estrus

TABLE 1

Summary of results showing how estrogen pretreatment facilitates the action of copper acetate in adult female rats

NUMBER OF RATS USED	PHASE OF CYCLE AND TREATMENT			PSEUDOPREGNANCY INDUCED		
	Estrus	Metestrus	Diestrus	No	Yes	Per cent yes
50	Cu			0	50	100
17		Cu		17	0	0
17			Cu	17	0	0
5	Sesame oil			5	0	0
17	Sesame oil	Cu		17	0	0
31	Estrone 4 to 12γ			31	0	0
7	Estrone 4γ	Cu		2	5	71
37	Estrone 8 to 12γ	Cu		0	37	100
6		Estrone 4 to 12γ		6	0	0
4		Estrone 4γ	Cu	4	0	0
8		Estrone 8 to 12γ	Cu	2	6	75
3		Estrone 16 to 24γ	Cu	0	3	100
7	Estrone 30 to 100γ			0	7	100

Cu denotes an intravenous injection of 0.1 cc. of a 1 per cent solution of copper acetate.
Sesame oil, subcutaneous injections ranging from 0.03 to 0.05 cc.
Estrone in sesame oil 400 gamma per cc. given subcutaneously.
Since these three phases of the estrus cycle come on successive days the interval between estrone and the copper injections was 18 to 20 hours.

maintains the cornification stage for the next day or two. These results indicate that an appropriate dose of estrogen makes the rat as responsive to an injection of copper solution at other stages of the cycle as it normally is at estrus.

A note of caution should be added here since it has been found that larger doses of estrone (30 gamma or over) given at the time of estrus will induce pseudopregnancy. In other experiments (not shown) single injections of 40 gamma given at estrus have caused the same cessation of cycles as was produced by the prolonged daily injections of 40 R. U. of estrone reported by Merckel and Nelson (4). Astwood and Greep (5) had reported that toxic materials would induce pseudopregnancy so a number of controls were given sesame oil

with and without subsequent injections of copper but the tests were uniformly negative as shown in table 1.

Since the completion of these observations on the rat the study has been extended by one of us (J. T. B.) to include the rabbit. Copper injected intravenously into the estrous rabbit induces ovulation and pseudopregnancy (6). However a series of tests have shown that anestrous rabbits do not ovulate after an intravenous injection of copper solution. Table 2 summarizes the results

TABLE 2

Summary of results showing that estrogen pretreatment of the anestrous rabbit makes it possible to induce ovulation by copper acetate

RABBIT	℞ ESTROGEN		℞ COPPER	OVULATION RESPONSE
	Date	Date	Date	
1			9/1	Negative
2			9/1	Negative
3			9/1	Negative
4			9/1	Negative
5			8/24	Negative
5	10/20	10/21	10/22	Positive
6			9/1	Negative
6	10/20	10/21	10/22	Positive
6			11/23	Negative
6	12/8	12/9	12/10	Positive
7	10/20	10/21	10/22	Positive
7			11/23	Negative
7	12/7	12/8	12/9	Positive
8	12/29	12/30		Negative
9	12/29	12/30		Negative
10	12/29	12/30		Negative
11	12/29	12/30		Negative
Controls (estrous rabbits)				
12			8/24	Positive
13			10/22	Positive
14			11/23	Positive
15			11/23	Positive
16	12/16	12/17		Negative
17	12/16	12/17		Negative

Dosage: Estrogen, 10,000 I.U. subcutaneously. Copper, 1 cc. of a 1 per cent solution of copper acetate intravenously.

obtained when anestrous rabbits were given an injection of 1 cc. of a 1 per cent solution of copper acetate. Ovulation was induced in all 4 control rabbits but in none of the 7 anestrous rabbits. However when the anestrous rabbits were given 10,000 I. U. of estrogen[4] subcutaneously on each of two successive days and then were given copper solution on the third day, ovulation occurred in each case (5 times in 3 rabbits). These rabbits were considered anestric on the

[4] This estrogen (40,000 I.U. per cc. in propylene glycol) was generously supplied by Dr. J. P. Schooley, Difco Laboratories Inc.

basis of having a tiny atrophic uterus judged by abdominal palpation and verified at laparotomy.

Discussion. The apparent enhancement of the sensitivity of the adult rat and the anestrous rabbit by pretreatment with estrogen may be an effect similar to that reported by Lane (7). He found that small doses of estrogen given for a short period of time seemed to stimulate the ovaries of the immature rat. Fevold et al. (8) and Frank and Berman (9) found that pretreatment with estrogen augments the ovarian response to gonadotropic extracts. Cytological studies of the pituitary indicate that the initial effects of estrogen may bring about a discharge of gonadotropic hormone (10).

There is also the possibility that estrogen pretreatment exerts its effect at least in part through the nervous system since the work of Brooks et al. (6) indicates that copper induces ovulation by an excitation of the central nervous system. Boling and Blandau (11) have shown that appropriate doses of estrogen and progesterone will induce a state of sexual receptivity in the guinea pig and rat. Ball (12) studied the effect of estrogen on sex behavior and concluded, "that the gonadal hormone does not organize the mating behavior pattern in the adult but merely activates a pattern already present." It seems possible, therefore that estrogen may sensitize certain neural mechanisms which normally function only at estrus. In our experiments it seems that copper is an effective stimulus only when some mechanism (possibly neural) has been activated by estrogen.

SUMMARY

1. Intravenous injections of 0.1 cc. of a 1 per cent copper acetate solution induce pseudopregnancy in rats if injected at the time of estrus. This dose of copper does not induce pseudopregnancy if injected during metestrus or diestrus.

2. Pretreatment with estrogen makes it possible to induce pseudopregnancy by injection of copper during metestrus or diestrus.

3. The dose range of estrogen which makes metestrous and diestrous rats equally as susceptible as the normal estrous rat to the stimulus of copper has been determined.

4. Pretreatment with estrogen makes it possible to induce ovulation and pseudopregnancy in the anestrous rabbit by an intravenous injection of copper acetate.

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THE EFFECT OF EXPERIMENTAL THYROID ABNORMALITIES ON APPETITE

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Our purpose in this investigation was to determine the composition of a diet which rats would choose if they could exercise choice, similarly as do people. After this dietary composition was determined by weighing the foods eaten by the rats, we then wished to make some animals hypothyroid by thyroidectomies, and others hyperthyroid by feeding them desiccated thyroid tissue. A recent clinical study on hyperthyroid patients (8) indicated uniformly low protein intake, as compared with a group of patients with supposedly normal thyroid activity.

There is an extensive literature which deals with the free-choice, or self-selection, or cafeteria-style method of feeding animals and children which we shall not review here (see references 1–7). Earlier studies have shown that within certain limitations, food selection as guided by appetite is a good method of promoting growth and health in animals and infants (7). Certain deficiency diseases have been found to be compensated for by modifications in dietary selection (6), but this is not always the case.

METHODS. 1. *Food selection.* We kept several groups of newly weaned male rats in individual round wire cages. Each rat was kept in an individual round wire cage, surrounded by six food cups. These cups contained casein, dextrose, leaf lard, baker's yeast (grown on molasses and killed), powdered agar, and Osborne and Mendel's salt mixture. We added a daily cod-liver oil supplement of one drop (1000 U.S.P. units of vitamin A and 100 U.S.P. units of vitamin D). Agar was added to supply bulk and is not further considered in this paper.

The food cups were weighed every four days, and filled again to a predetermined weight in grams. We used 100 cc. glass beakers as food cups, and avoided spillage by a hollow metal cylinder insert. In this cylinder we soldered a downward-sloping shelf, with a $\frac{3}{4}$ inch hole in the middle.

2. *Basal metabolic rates* (*BMR's*). The apparatus used was of the type which measures oxygen consumption. The animal container was a large glass desiccator jar, in which the animal sat on a wide-mesh wire screen. The bottom of the jar contained saturated salt water, which deodorized excreta and kept the humidity relatively low. The animal received oxygen from a rectangular float which moved a pointer on a large scale, so that the cubic centimeters of oxygen used could be read off. Soda lime was placed in a wire "collar" surrounding the animal for the absorption of carbon dioxide. During the routine tests, the carbon dioxide concentration in the animal chamber was about 0.10 per cent, and the oxygen concentration was about 21.5 per cent. Three such entire

apparatus were set in soft rubber and housed in a noiseless constant temperature chamber. All readings could be made through a glass front, without opening the chamber. The rats were exposed to fairly bright lights during the tests, since this seemed to keep them more quiet.

Rats were fasted 18 hours prior to a BMR test. A test consisted of four measurements of oxygen consumption while the animal was quiet, each for a period of 15 minutes. The average of the two lowest readings was used as the basis for calculation. We calculated all our BMR's as calories per square meter of body surface per 24 hours, using the following formula:

$$\frac{24 \times \text{cc. } O_2 \text{ used/hr.} \times 4.825 \times \text{correct. T. \& P.}}{9.1 \sqrt[3]{\text{Wt. of animal}^2}} = \text{Cal./sq. m./24 hr.}$$

The variation or "error" on repeated tests with the same animal was about 6 per cent by the above method.

3. *Thyroidectomies on rats.* A successful complete thyroidectomy was indicated by a notably coarser hair coat, by sluggishness, cessation of growth and by a drop in the BMR. Only about half of the thyroidectomies performed met these criteria over a period of months; the remainder showed an initial effect which wore off after a few weeks, and some of these animals later became very hyperactive.

4. *Hyperthyroid rats.* It was relatively easy to make animals hyperactive, with cessation of growth and a rise of 30 per cent to 50 per cent in the BMR. We mixed 100 mgm. of desiccated thyroid (Armour's) into a solution of 25 per cent cane sugar, and fed each rat a half cubic centimeter (i.e., 50 mgm. of thyroid) every two or three days, depending on how definitely hyperthyroid the animal became. Rats like sugar water, and licked this thyroid mixture directly off the tip of a tuberculin syringe.

RESULTS. 1. *General character of self-selection.* The survival of the species is proof that rats are able to find and select a diet in nature which supports growth and reproduction. The food elements found "in nature", however, are very likely to be mixtures of various constituents, and thus survival of the race does not answer the question whether the chemical detectors at the oral end of the organism are adequate to select an adequate diet from among chemically pure foods.

In a short preliminary experiment we fed 13 rats with four feeding cups in each cage, the cups containing casein, dextrin, lard, and a mixture of equal parts of yeast and salt mixture. After one month, 5 of these rats looked healthy and gained weight. The other 8 rats looked sick and lost weight. The intake of dextrin and lard in the two groups was not very different, but the casein intake was dramatically low for the sick group. This absence of protein appetite was possibly due to a low intake of yeast, as seen in table 1. This table presents the average intake per rat in grams per day of the two groups of rats.

We separated the salt mixture and the yeast into separate feeding cups, and in further experiments only about 1 rat in 20 failed to select a diet which supported growth. The character of self-selection in a typical normal rat over a

period of several months is illustrated in figure 1, and is discussed later. Figure 1 gives a curve of total caloric intake at the top; then three curves of caloric intake as protein, carbohydrate and fat. This rat was 3 months old at the beginning, and the dates give the time of year and duration of study. Below is a growth curve and the curve of normal BMR variation.

2. *Growth curves.* We charted the growth of individual rats, as well as of groups of rats, in order to have a constant objective criterion of the general welfare of our animals. The first indication of dietary insufficiency (because of poor selection or other reasons) was frequently a change of slope in the growth curve.

TABLE 1

Average food intake per rat per day

	CASEIN	DEXTRIN	LARD	SALTS AND YEAST	TOTAL
	grams	*grams*	*grams*	*grams*	*grams*
Healthy rats	2.3	3.3	2.1	1.1	8.8
Sick rats	0.3	3.0	1.6	0.6	5.5

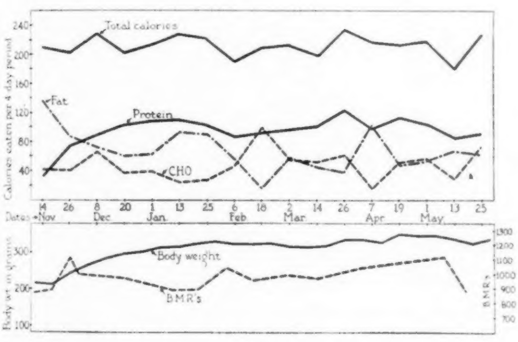

Fig. 1

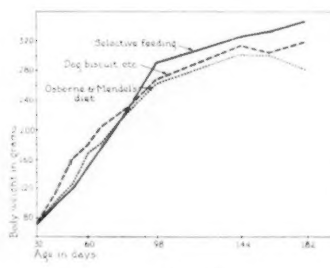

Fig. 2

Fig. 2. Study of growth curves in 3 groups of litter-mate controls with 5 rats in each group

We found that the great majority of rats selected a diet which promoted normal growth. Figure 2 shows growth curves of 3 groups of litter-mate controls. One group received the standard Osborne and Mendel diet mixture, another was fed Purina dog biscuit and various other foods used for stock animals in our laboratory, and the third group was on selective feeding. During the first one and a half months the selecting rats did slightly less well than the others, but in the long run the selected diet seemed somewhat superior in promoting growth. The results of this experiment prompted us to use a larger number of animals over a longer period of time—an experiment that is still in progress.

Figure 3 shows the general trend of the individual growth curves of six normal

rats kept on selective feeding for almost a year, as well as growth curves of a typical hypothyroid and of a typical hyperthyroid rat.

3. *Age differences in food selection.* In the course of our work we discovered that rats seem to undergo a change of appetite at the age of about 4 months. This is illustrated by the data in table 3. The age of 4 months also marks a decrease in rate of growth (fig. 3). In order to make possible comparisons among rats of various ages and sizes, all our data below was calculated in terms of grams or calories eaten *per 100 grams body weight* per 4-day period. Then

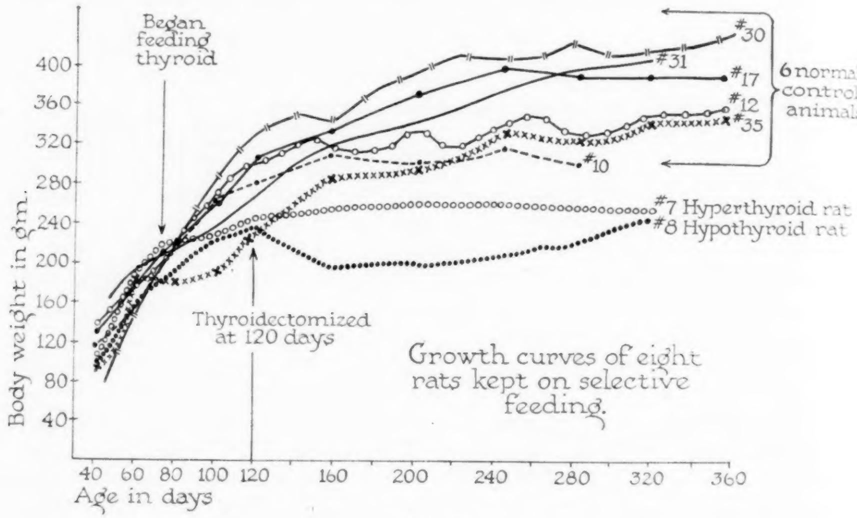

Fig. 3

TABLE 2

Average BMR's in cal./sq. m. body surface/24 hours

	BEFORE 120 DAYS (ALL ARE NORMALS HERE)	AFTER 120 DAYS
Straight normals	889	862
Hypothyroids	891 (normal period)	736 (hypo-period)
Hyperthyroids	780 (normal period)	1060 (hyper-period)

we chose the age of 120 days as an arbitrary division between "young" and "old" rats, and made sure that all our comparisons took this factor into consideration.

4. *Basal metabolic rates.* We used three groups of rats, with 6 rats in each group. Up to the age of about 100 to 120 days, all 3 groups were normal animals. After the age of about 120 days, we selected 6 healthy animals as normals (see growth curves in fig. 3), 6 others from our thyroidectomies as good hypothyroid rats, and the remaining 6 were our best hyperthyroid rats. Table 2 presents average BMR's for these 3 groups; in any one group the *same animals*

studied before the age of 120 days are also used in data for the period after the age of 120 days.

It will be noted that the strictly normal group experienced a drop in BMR of 3 per cent after the age of 120 days. The hypothyroids were 17 per cent lower after thyroidectomy than before. Finally, the hyperthyroid group had a 36 per cent higher BMR after the feeding of thyroid tissue than before. The clinical symptoms of hypothyroidism and hyperthyroidism correspond quite closely to the BMR. While the hypothyroids seemed sluggish, the hyperthyroids often made themselves conspicuous by violently jumping around in

TABLE 3

Average grams of food eaten per rat per 100 grams body weight per 4-day period

FOOD ARTICLE	BEFORE 120 DAYS (ALL NORMAL HERE)			AFTER 120 DAYS (POST-OP., ETC.)		
	Straight normals	To be hypo-	To be hyper-	Straight normals	Hypo-thyroids	Hyper-thyroids
	grams	*grams*	*grams*	*grams*	*grams*	*grams*
Casein	5	5	4	6	6	6.5
Dextrin	7	8	12.5	5	5	7
Lard	3	2.5	1	1	1	2
Yeast	3	5	5.5	2	1.5	3
Salt	0.3	0.6	0.2	0.2	0.3	0.2
Total	18.3	21.1	23.2	14.2	13.8	18.7

TABLE 4

Average food intake of 3 groups of rats, in per cent, calculated on basis of weight of food in grams

FOOD ARTICLE	BEFORE 120 DAYS (ALL NORMAL HERE)			AFTER 120 DAYS (POST-OP., ETC.)		
	Straight normals	To be hypo-	To be hyper-	Straight normals	Hypo-thyroids	Hyper-thyroids
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Casein	27	24	17	42.5	44	35
Dextrin	38	38	54	35	36	37
Lard	17	12	4	7	7	11
Yeast	16	24	24	14	11	16
Salt	2	2	1	1.5	2	1
Total	100	100	100	100	100	100

their cages. Several such hyperthyroid rats learned to pull our non-spill feeders apart.

5. *Composition of diets selected.* In reporting our data, we have converted all figures to apply per 100 grams of the animals' body weight, so that we can compare the feeding of young and of older animals. Table 3 shows the intake, in grams, averaged for the three groups of 6 rats each, and calculated for a 4-day period. For ease in further interpretation, the data in table 3 have been converted into percentages, as seen in table 4.

Finally, table 5 presents the percentages of *caloric* intake as protein, carbohydrate, and fat. Tables 3, 4 and 5 all contain data based on the same 18 rats.

Discussion. An examination of tables 3, 4 and 5 yields several observations. (Note that data in table 3 are per 100 grams body weight.) It is seen from table 3 that there is a relative decrease in food intake with increasing age. This decrease amounted to 22 per cent in our normal rats, to 35 per cent in thyroidectomized rats, and to only 19 per cent in the hyperthyroid rats. Actually, these hyperthyroid rats ate a lot more food per 100 grams body weight than did either the normals or hypothyroids (table 3).

A decrease in appetite for lard with increasing age is seen in these tables, at least in the normal and hypothyroid rats. The increase in lard consumption by hyperthyroid rats is largely due to one rat, which ate much lard after being fed thyroid tissue.

These tables show an unexpectedly high protein intake. As seen in table 5, young rats selected 28 per cent to 32 per cent protein (this includes the protein fraction of yeast). Older rats even showed a marked increase in this protein

TABLE 5

The percentages of caloric intake as protein, carbohydrate and fat

DIETARY CONSTITUENT	BEFORE 120 DAYS (ALL NORMAL HERE)			AFTER 120 DAYS (POST-OP., ETC.)		
	Straight normals	To be hypo-	To be hyper-	Straight normals	Hypo-thyroids	Hyper-thyroids
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Protein	30	32	28	46	46	38
Carbohydrate	39	44	63	39	39	41
Fat	31	24	9	15	15	21

feeding, to the point where almost half their diet consisted of protein. This was not due to illness, because all animals reported here were consistently healthy, and the normals had a good coat of hair at all times. This experimental result raises a question as to why over 22 per cent protein should impair the appetite of white rats, as has been reported (9). The high intake of protein is the more remarkable in view of the apparent dislike rats have for casein. However, this is explained in part by the fact that some of the protein intake was in the form of yeast, which rats like.

The relatively high intake of yeast by these rats is interesting and contrary to earlier reports (10). For some 4-day periods certain rats averaged over 5 grams of yeast intake per day, and they not uncommonly ate 3 or 4 grams of yeast per day. Table 3 shows that the thyroidectomized animals demonstrated the greatest drop in yeast appetite.

The appetite of rats for salt mixture has varied somewhat in our experience with selective feeding, and has been greater in some groups of young rats than is shown here. However, older rats uniformly prefer a low salt intake, and they usually eat slightly less than 2 per cent salt in their selected diet (table 4).

The appetite of rats for dextrin resembles their appetite for lard in that these constituents are selected with peculiar variations. A rat may suddenly reverse

its selection from a low carbohydrate and high fat diet, to a low fat and a high carbohydrate diet; some weeks or months later another reversal of selection may occur (fig. 1). Such variable feeding on dextrin and lard is reflected in the variations among the three groups before the age of 120 days, when the rats are still all normal (tables 3 and 4). These observations resulted from our study of individual animals over a period of several months. Other studies in self-selection with rats have not been continued for such long periods of time (1, 4, 6).

In closing this discussion some comparison should be made between the diets selected by our rats and a well-known standard diet, such as that of Osborne and Mendel. We have found that the latter diet does not support good growth in rats after they have been raised on it to the age of 4 or 5 months. Possible deficiencies may be suggested by the comparison in table 6.

It is readily seen that the young rats select a diet more nearly like that of Osborne and Mendel than do the older rats. The low protein, high fat and salt content of the standard diet seem to make it unsatisfactory for older rats.

TABLE 6

Constituents of the standard Osborne and Mendel diet, of the self-selected diet of our 18 young rats, and of our 6 older rats, respectively

FOOD ARTICLE	OSBORNE AND MENDEL DIET	DIET SELECTED BY YOUNG RATS	DIET SELECTED BY OLD RATS
	per cent	*per cent*	*per cent*
Casein	18	22	42.5
Dextrin	52	44	35
Lard	24	10	7
Yeast	3	22	14
Osborne & Mendel salt mixture	3	2	1.5

CONCLUSIONS

1. Selective feeding, using relatively pure constituents, will support good health and growth in most rats for a period of over one year.
2. All food constituents should be kept in separate feeding cups, rather than having some mixed together (such as yeast and salt).
3. The diet selected by rats varies according to age; and the critical age dividing "young" and "old" rats is about 4 months.
4. Older rats eat less food per 100 grams body weight than do young rats.
5. Young rats select more fat and salt than do older rats, while the latter eat more protein. However, all our groups have eaten much more protein than is allowed in the Osborne and Mendel diet.
6. The alternate intake of fat and carbohydrate is so definite in some rats as to suggest that they "eat for calories."
7. Six rats were made hypothyroid, 6 others were made hyperthyroid, and 6 were kept as normal controls. The thyroidectomized rats showed a markedly greater decrease in food intake than did the normals, while the hyperthyroid rats ate much more food than either of the other two groups. No characteristic qualitative differences in food selection were noted due to thyroid abnormalities.

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DEPRESSIVE EFFECTS PRODUCED ON APPETITE AND ACTIVITY OF RATS BY AN EXCLUSIVE DIET OF YELLOW OR WHITE CORN AND THEIR CORRECTION BY COD LIVER OIL[1]

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In terms of the actual work output of individual organisms very little is known regarding the energy value of various foodstuffs. In the series of which the present experiment forms a part an effort is being made to throw more light on this phase of the nutritive value of foods.

In previous experiments measurements were made of the spontaneous work output (revolutions of a drum) of rats kept on a diet in which dextrose constituted the sole source of nourishment (1). On this diet the rats lived an average time of 36 days and did not show any signs of specific nutritional deficiencies, though they were markedly emaciated. Of special interest in the present connection is the fact that the rats remained very active, averaging 10 to 12 miles per day in the revolving drum for the first 20 days. When given access to a 0.02 per cent solution of thiamine hydrochloride the rats on dextrose lived slightly more than twice as long, averaging 74 days. The only definite deficiency symptom shown during this time was a constant cornification of the cells of the vaginal smears, after an average time of 51 days, presumably indicating a lack of vitamin A. Here again the rats remained very active, averaging 6 to 8 miles per day up to 60 days on the diet. Clearly, dextrose, either alone or supplemented with thiamine hydrochloride, serves as an excellent source of energy. Similar studies have been made using maltose, sucrose, levulose, galactose and lactose (2, 3).

In the present experiments this same technique has been applied to the study of the energy value of white and yellow corn. Measurements of activity have been made, as well as observations on the effects produced on appetite and water intake, body weight, vaginal smear cycles, and general health.

METHODS. Eight female rats were placed in separate activity cages at an average age of 45 days; for the first 15 days they were given free access to our stock diet and tap water. At 60 days of age and an average body weight of 135 grams, these rats were placed on an exclusive diet of either freshly ground yellow or white corn[2] and tap water. Eighty-five days later they were given access also to cod liver oil. Another 40 days later, that is at an average age of 185 days, they were killed and autopsied.

[1] Carried out under grants from the Corn Industries Research Foundation, New York City and the Committee for Research in Endocrinology of the National Research Council, Washington, D. C.

[2] The yellow hybrid corn g-94 and the white hybrid corn g-527W were kindly furnished by Funk Brothers Seed Company.

The cages were composed of two parts: a living compartment 11 x $3\frac{1}{2}$ x 5 inches and a revolving drum $12\frac{1}{2}$ inches in diameter and 6 inches wide, separated by a metal partition with an opening 3 inches in diameter. The living compartment, which contained a food cup with a shield built to eliminate all spillage, and a 100 cc. graduated inverted water bottle, was made as small as possible in order to force the rat to expend most of its energy in the drum, where it could be measured by means of a cyclometer. The cod liver oil was later offered in an added 30 cc. graduated bottle.

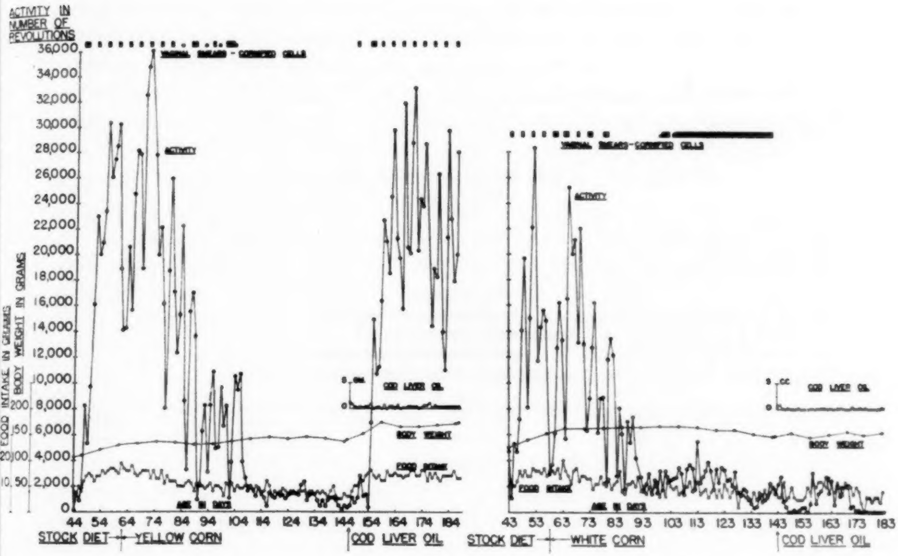
For the reason that coprophagy may play a particularly important part in single food choice experiments, special precautions were taken to prevent easy access to the feces. In the living compartment, a $\frac{1}{3}$ inch wire mesh bottom permitted the feces to drop through freely to a sawdust pan several inches below and beyond the reach of the rat. In the revolving drum, which was made of $\frac{1}{4}$ inch wire mesh, a space of $\frac{1}{4}$ inch between the drum and the central partition permitted the feces to drop through freely to the pan, well beyond the rat's reach. The results of previous experiments have shown that this method limits coprophagy sufficiently to give quantitatively reproducible results (1).

Records were made daily of running activity, food and water intake, and vaginal smears, and weekly of body weight. The rats were carefully examined at weekly intervals for specific signs of nutritional deficiency.

Results. *Yellow corn.* Figure 1A gives the record of one of the 4 rats on yellow corn. The abscissae show age in days, the ordinates running activity in revolutions of the drum, food intake and body weight in grams. This rat was placed in the activity cage on our stock diet at an age of 44 days, changed to the yellow corn diet at 62 days, and given access to cod liver oil at 146 days of age. During the last 5 days on the stock diet the daily running activity reached an average level near 24,000 revolutions and food intake a level near 16 grams. On the yellow corn diet activity remained on essentially the same level for 20 days, then decreased, first to a level near 8,000 revolutions per day, and finally, between the 136–146th day period, to a level around 1,000 revolutions per day. For the first 10 days the intake of corn approximately equalled the previous intake of stock diet. Thereafter, up to the 146th day, the intake decreased at a slow but steady rate, finally reaching a level near 7 grams per day. Body weight increased slightly during the 80 days on the corn diet. Vaginal smears (cornified cells shown at the top of the chart) showed regular 4 to 5 day cycles for the 15 days on the stock diet and for the first 20 days on the corn diet. Thereafter the cycles became irregular for 20 days and then disappeared altogether, leaving only a diestrous picture of leucocytes and nucleated epithelial cells. Otherwise the rat did not show signs of any specific nutritional deficiency at this time. Cod liver oil offered on the 146th day was taken in moderate amounts for the first day, then only in minimal amounts. The cod liver oil ingestion had immediate and profound effects on activity, corn intake, body weight and vaginal smears. Within 18 days the average daily activity had increased from a level around 1,000 revolutions to 22,000 revolutions (13.5 miles), approximately the same as that on the stock diet; the average daily corn intake increased from

7 to 14 grams, and the body weight from 140 to 174 grams; within 4 days the vaginal smears again showed regular 4 day cycles of cornification, coinciding with the peaks of running activity.

Table 1A summarizes the results of the observations made on the 4 rats on yellow corn. The average daily activity decreased from 19,969 revolutions for the first 10 days on the corn diet to 2,139 revolutions for the 70–80th day period, and increased again to 15,803 for the 30–40th day period on cod liver oil. All 4 rats became more active within 3 or 4 days after the addition of the cod liver oil. The average daily corn intake decreased from 15.4 grams for the first 10 days to 7.5 grams for the 70–80th day period, and increased again to 11.3



A B

Fig. 1

grams for the 30–40th day period on cod liver oil. All four rats showed a detectable increase in food intake after the second day on cod liver oil. Body weight increased from an average of 132 grams at the start of the corn diet to 143 grams on the day cod liver oil was started. (During the same interval control rats on the stock diet increased to 189 grams.) At the end of the 40 day period on cod liver oil the average body weight had increased to 168 grams.

Figure 2 summarizes the effects produced on vaginal smears, showing only the cornified cells. All 4 rats on yellow corn showed quite regular 4 to 5 day cycles for the 15 days on the stock diet and for the first 20 days on corn. From then on, until the cod liver oil was offered, the cornified cells gradually disappeared from the smears, leaving only leucocytes and epithelial cells. Within 4 days of

the addition of cod liver oil, 3 rats showed their first day of cornified smears, the fourth showed it 4 days later. Thereafter during the 40 day period all 4 rats, with the exception of one rat which missed 2 cycles, showed 9 regular 4 day cycles. It is also noteworthy that the peaks of running activity coincided as they do in normal animals with the days of cornification.

Just prior to the addition of the cod liver oil 3 of these animals were reported in excellent condition and the fourth was in good shape except for slight rusting of the back of the head and neck. When they were killed, 40 days later, their appearance differed little from that of normal controls. Body weight averaged only 21 grams less, eyes were large and clear, teeth were normal, paws and tails were clean; however, the hair was yellow and matted over the hind quarters of all 4 animals. At autopsy there were no gross deviations from the normal, except a complete absence of thymus tissue, whereas this gland weighed an average of 176 mgm. in normal controls. As seen in table 2A, the weights of the other endocrine glands all fell within normal limits, with the exception of an unexplained increase in size of the ovaries, which averaged 42 and 40 mgm. for

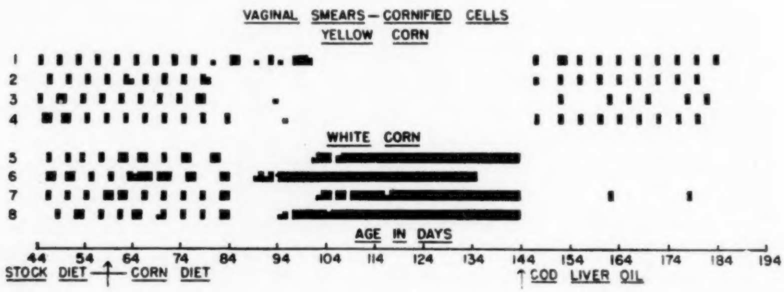


Fig. 2

the left and right glands respectively, as compared with 36 and 32 mgm. in the controls.

White corn. Figure 1B gives the record of one of the 4 rats on the single food choice of white corn. For the first 100 days of the experiment, that is, until the cod liver oil was added, the activity, food intake, and body weight records are essentially the same as those of the rat on yellow corn shown in figure 1A. The vaginal smears, however, differed markedly. In contrast to the constant diestrous smears of the rat on yellow corn, the smears of this rat showed cornified cells and no leucocytes or epithelial cells for the last 35 days before cod liver oil was added. Also in contrast with the yellow corn-fed rats, the cod liver oil, which was taken in minimal amounts, had no effect on activity, only a temporary if any effect on food intake, and body weight showed only a very small increase. A most striking effect was produced, however, on the vaginal smears. Within 24 hours all cornified cells disappeared, leaving only epithelial cells and leucocytes, but still giving no evidence of cyclic changes.

Table 1B summarizes the results of the observations made on the 4 rats on

white corn.[3] On the corn diet the average daily activity of the 4 rats decreased from 16,203 revolutions for the first 10 days to 2,116 for the 70–80th day period. On the cod liver oil one of the surviving 3 rats became slightly more active again, while the other two became progressively more inactive. The average daily intake of white corn decreased from 14.8 to 5.5 grams. On the cod liver oil 2 rats showed an increased, one a decreased food intake. The average body weight decreased from 138 grams on the first day of the corn diet to 126 grams on the 83rd day. On the cod liver oil one rat showed a marked increase in weight and one a moderate increase, while one maintained its weight. Figure 2 shows that the cornified cells disappeared from the vaginal smears on the day after cod liver oil was made available. Thereafter only one animal showed any cornified cells, then only on 2 days. Thus, the records of the yellow corn-fed rats before the addition of cod liver oil were very similar to those of the white corn-fed group after the oil was added.

Before the cod liver oil was added, 3 of the 4 animals showed deficiency symptoms of eyes, teeth or hair, and when they were killed 40 days later they were very clearly distinguishable from the normal controls and from the yellow corn-fed rats, mainly by their smaller size, but also by a marked yellowing and roughening of the coat, by their spectacle eyes, and by the fact that all except one had poor teeth: in 2 cases both upper incisors were broken off and the lowers were abnormally long. Autopsy revealed no gross abnormalities except the complete absence of thymus tissue noted also in the yellow corn-fed group, but all endocrine glands were found to be markedly smaller than those of normal controls. In particular, whereas the thyroids, adrenals, and ovaries were decreased approximately in proportion to the lower body weight, the uteri were found to be relatively far smaller, weighing approximately 20 per cent as much as those of the controls.

Discussion. Corn is a cereal grain of such great agricultural importance that it is only natural that it should have been thoroughly studied from a nutritional standpoint. It has long been known that zein, the principal corn protein, is deficient in the amino acids, tryptophane, tyrosine and lysine (4, 5). In addition, corn is known to be a poor source of calcium (6). In 1919 Steenbock compared the nutritive value of various species of corn, and pointed out the higher vitamin A content of the yellow varieties (7). The vitamin B complex, on the other hand, is apparently present in adequate amounts for normal growth in both yellow and white varieties (8). Since pellagra is so very common in regions where corn constitutes a large proportion of the diet, and since a deficiency of nicotinic acid is known to play a major rôle in the production of this disease syndrome, it is not astonishing to find that corn is a poor source of this vitamin (9). Steenbock and Coward also pointed out that the vitamin D content of corn is low (10). Apparently, like other cereal grains, it is relatively rich in vitamin E (11).

However, when rats were kept on an exclusive diet of either white or yellow corn from 60 to 145 days of age, none of these deficiencies became sufficiently

[3] One of the rats died after 76 days on the diet.

TABLE 1

RAT NO.	AVERAGE DAILY RUNNING ACTIVITY IN REVOLUTIONS OF DRUM			AVERAGE DAILY CORN INTAKE IN GRAMS			BODY-WEIGHT IN GRAMS		
	First 10 days on corn diet	70–80th days on corn diet	30–40th days on cod liver oil	First 10 days on corn diet	70–80th days on corn diet	30–40th days on cod liver oil	At start of corn diet	At end of 80 days on corn diet	At end of 40 days on cod liver oil
				A. Yellow corn					
1	23,162	1,024	22,201	16.2	7.7	14.0	134	140	174
2	14,255	1,241	17,872	13.7	7.3	10.0	124	146	157
3	22,560	4,401	14,816	14.9	8.2	9.5	122	142	162
4	19,898	1,889	8,324	16.6	6.9	11.7	147	145	178
Average...	19,969	2,139	15,803	15.4	7.5	11.3	132	143	168
				B. White corn					
5	12,369	1,287	380	15.4	5.9	7.3	152	150	151
6*	23,734	2,371		15.7	4.7		135	114	
7	16,069	1,969	5,964	13.5	5.8	10.1	132	116	174
8	12,640	2,837	1,117	14.4	5.5	5.0	133	124	144
Average ..	16,203	2,116	2,487	14.8	5.5	7.5	138	126	156

* Animal died after 76 days on white corn diet.

TABLE 2

Autopsy findings

Endocrine weights in milligrams

RAT NO.	BODY WEIGHT	ADRENAL LEFT-RIGHT	UTERUS	OVARIES LEFT-RIGHT	THYROID	THYMUS	TEETH
				A. Yellow corn			
	grams						
1	174	35–30	411	48–49	11	Absent	Good condition
2	157	34–28	348	35–32	16	Absent	Good condition
3	162	24–26	231	38–37	14	Absent	Good condition
4	178	25–15	421	45–42	14	Absent	Good condition
Average............	168	29–25	353	42–40	14		
				B. White corn			
5	151	25–23	131	28–38	16	Absent	Upper teeth broken off
7*	174	20–24	182	30–34	16	Absent	Poor condition
8	144	13–14	92	15–11	11	Absent	Poor condition
Average...........	156	19–20	102	24–28	14		
				C. Regular food			
Controls 150 days .	189	26–26	498	36–32	17.4	176	Good condition

* Animal 6 died after 76 days on the white corn diet and no autopsy was obtained.

marked to be obvious unless records were kept of the activity and vaginal smears. Thus, in both groups, the weight was essentially maintained though there was little or no growth; there were no evidences of dermatitis and no gross skeletal changes. Only during the last quarter of the period did the white corn-fed rats begin to show the spectacle eyes and roughening of the fur characteristic of an early vitamin A deficiency, as well as poor occlusion of the incisors. On the other hand, during the first 25 days of the diet both groups of animals showed a loss of spontaneous activity of nearly 70 per cent, and the vaginal smear cycles were lost: the white corn-fed rats showed persistent cornification, while the yellow corn-fed animals went into a state of constant diestrous.

But the striking difference between the two groups was only brought out when cod liver oil was made available. Whereas this addition had little or no obvious effect on the food intake, body weight, and activity of the white corn-fed rats, it produced in them an almost immediate change in the vaginal mucosa from a state of constant cornification to one of constant diestrous. In contrast, the yellow corn-fed rats showed a marked general improvement almost immediately following the ingestion of the cod liver oil: first the food intake and body weight began to increase, then normal 4 day estrous cycles reappeared, and later (approximately 7 days after the oil was added) the activity increased abruptly.

It is not clear at this time what factors in the cod liver oil are responsible for the various changes: both vitamins A and D must be taken into consideration, as well as the various fatty acids, sterols, and other, perhaps unknown, constituents. The fact that the yellow corn-fed rats returned to normal cycles of vaginal cornification, and gained weight, and became more active when cod liver oil was added to their diet, raises the question whether all three changes were brought about by the same factor or whether there were a number of substances involved. The data so far available are not sufficient to answer this question.

On the assumption that for the purposes of relatively short term experiments, yellow corn is lacking primarily in one substance, one can postulate further concerning the deficiencies of white corn. In the first place, there is almost certainly a deficiency of vitamin A, which is corrected by the fish oil supplement. In the second place, it seems likely that the white corn is deficient also in the same substance lacking in yellow corn and supplied by cod liver oil, whether this is vitamin D or some other factor. Further, since the addition of cod liver oil to the diet of the white corn-fed rats still leaves them in a state of obvious nutritional deficiency, as indicated by the limited appetite and growth, inactivity, diestrous smears and general appearance, there would seem to be lacking in the white corn some factor present in yellow corn and not supplied by cod liver oil.

SUMMARY

1. Female rats on an exclusive yellow corn diet for a period of 85 days showed a gradual loss of appetite, a scarce maintenance of starting body weights, a great loss of activity, and development of a diestrous condition of the vaginal mucosa. They did not, however, show any other signs of specific nutritional deficiency in this time.

2. Female rats on an exclusive white corn diet for a period of 85 days showed a similar picture, with the exception that after 45 days the cells of the vaginal smears became constantly cornified, and after about 76 days the upper teeth became worn and the lower teeth overgrown. They also showed deficiency symptoms of the eyes and hair.

3. Cod liver oil offered the rats on the yellow corn diet was taken in moderate amounts for the first day and then in minimal amounts, resulting in an almost immediate increase in appetite, body weight and activity. Regular 4 day cycles also reappeared in the vaginal smears within 4 days. Within 20 days the rats had practically reached their original high running levels.

4. Cod liver oil offered the rats on the white corn diet was taken in minimal amounts, producing almost no effect on activity, a small effect on appetite, and only a slight increase in body weight. Within a day, however, the vaginal smears were changed from a condition of constant cornification to one of constant diestrous.

5. In white corn there is almost certainly a deficiency of vitamin A, as well as of the factor lacking in yellow corn, both of which are corrected by the cod liver oil supplement. Moreover, there is evidence to indicate that yellow corn contains some factor lacking in white corn and not present in cod liver oil.

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THE EFFECT OF URANIUM POISONING ON PLASMA DIODRAST CLEARANCE AND RENAL PLASMA FLOW IN THE DOG[1]

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The value of the clearance method in the study of renal physiology is now clearly recognized. The validity of the inulin clearance (C_I) as a measure of glomerular filtration and of the diodrast clearance (C_D) as a close approximation to the plasma flow to the active secretory and excretory portions of the normal kidney (effective plasma flow) has been established for several species, chiefly the dog and man. The uninephrectomized dog with the remaining kidney explanted is a particularly satisfactory subject for studies of clearance and renal blood flow (RBF) or plasma flow (RPF) since the latter may be calculated directly from the clearance and arterio-venous difference of any test substance. This procedure has been used in the study of the relation between C_D and RBF in the normal dog kidney (White, 1940a; Corcoran et al., 1941) and in the damaged kidney (White, 1940b; White et al., 1941).

In a previous paper (Hayman et al., 1939), the effect of uranium on the dog kidney was discussed and data were presented to show that the decrease in C_I and creatinine clearance (C_C) was not due to a decrease in RBF. The low clearances and extractions seemed most satisfactorily explained, considering the histological as well as the analytical data, on the basis of back diffusion of inulin (I) and creatinine (C) in the damaged tubules. The greater decrease in C_C than in C_I was presumed to be the result of the greater back diffusion of the smaller C molecule. During the course of a study of the relation between C_D and RBF in the dog kidney damaged by a variety of means, uranium poisoning has been reinvestigated and the method has been extended to include the use of diodrast (D) as a test substance.

Methods. Trained dogs were used, maintained on the usual kennel rations including meat. One kidney was explanted to make the renal vein accessible for venipuncture. Following recovery from this operation, the other kidney was removed. Experimental studies were begun no sooner than three months following the second operation.

Two control experiments were performed on each dog before it was given a subcutaneous injection of 2 mgm. of uranium acetate per kilo of body weight. Following the administration of uranium, the experiments were repeated at the height of the effect (about five days) and at varying intervals thereafter until

[1] Supported by a grant from the Commonwealth Fund.

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substantial recovery had occurred. One dog, no. 4, was sacrificed after the five-day period for histological examination of the kidney.

Clearance and extraction data were obtained as follows. An intravenous infusion of normal saline solution was started and maintained throughout the experiment at a rate of about 2 cc. per minute to insure adequate urine flow. C, I and D in saline solution were injected subcutaneously in two sites in a total volume of 30–50 cc. For control experiments, 0.1 gram of C, 0.2 gram of I, and 0.175 gram of D (0.5 cc. of 35 per cent solution) per kilo of body weight were used. Following kidney damage, these quantities were reduced one-half.

Fifteen minutes following the injection, the bladder was emptied and washed with normal saline solution through an in-dwelling catheter. Three 20-minute urine collections were made, the bladder being washed with two 10 cc. portions of saline solution at the end of each period. Blood samples were taken from the jugular vein ("arterial") and renal vein ("venous") as near to the mid-points of the urine collection periods as possible. These periods were used for D clearances only. The plasma D level during this 60-minute interval remained very nearly constant at a level of about 5 mgm. per 100 cc. or less. D was determined in arterial and venous whole blood, arterial plasma and urine, giving data for the calculation of both whole blood and plasma C_D, whole blood extraction and RBF.

Following these three periods, 1.5–2.0 cc. of 35 per cent D solution per kilo of body weight were injected intravenously. Beginning 15 minutes later, four additional clearance periods were obtained as above. Arterial plasma was analyzed for D, I and C, venous plasma for I and C, urine for D, I, C and urea (U). The pooled remainders of arterial plasma were utilized for albumin and U determinations. The D, I, C and U plasma clearances, RPF (using both I and C extractions) and Tm were then calculated from the data. Other calculations are explained in the discussion. Hematocrit readings were obtained on a sufficient number of blood samples to make possible the interconversion of RPF and RBF.

During this 80-minute interval, the plasma I level remained very nearly constant at 5–10 mgm. per 100 cc. The plasma C level fell slowly, the D level more rapidly but the rate of fall in both cases was so nearly linear that no significant error was introduced by assuming the interpolated plasma concentration at the mid-point of the urine collection period to be equal to the mean concentration throughout the period.

Diodrast was determined in both plasma and whole blood by the method of Alpert (1941) with the application to the whole blood filtrate analyses of a correction factor based on the hematocrit reading (Bobey and Price, 1942). Inulin was determined by the method of Alving, Rubin and Miller (1939), using the Cenco-Sheard spectrophotelometer for the color readings. Creatinine was determined in Folin-Wu filtrates as the alkaline picrate, by a standard method slightly modified for use with the Spectrophotelometer. Plasma urea was determined by the method of Van Slyke and Kugel (1933), urine urea by Van Slyke's urease method (1937). Albumin was calculated as N × 6.25 after Kjeldahl analysis of the globulin-free filtrate obtained with 22 per cent sodium sul-

TABLE 1

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
		CLEARANCE					EXTRACTION RATIO								
DOG	TIME AFTER URANIUM	Inulin plasma	Creatinine plasma	Urea plasma	Diodrast plasma	Diodrast blood	Inulin plasma	Creatinine plasma	Diodrast blood	AVERAGE RPF	AVERAGE RBF	C_D/RPF $\frac{(7)}{(11)}$	Tm	TUBULAR C_D	TUBULAR EXTRACTION $\frac{(15)}{(11)}$
		cc./min.	*cc./min.*	*cc./min.*	*cc./min.*	*cc./min.*				*cc./min.*	*cc./min.*		*mg.I/min.*	*cc./min.*	
1	Control	33.5	31.9				0.322	0.254		115	191				
	5 days	7.7	5.7				0.077	0.053		105	173				
	7 weeks	19.4	15.8				0.221	0.195		85	151		2.9		
	14 months	32.4	32.0	22.1	78	96	0.275	0.325	0.607	105	162	0.74	5.1	51.0	0.49
2	Control	47.8	47.8	27.2	128	148	0.300	0.293	0.611	162	254	0.79	7.2	86.0	0.53
	5 days	18.1	15.2	11.9	13.8	15.5	0.077		0.055	214	319	0.06	−1.0	−2.4	−0.01
	12 days	24.1	22.0	16.1	44.4	52.4	0.190	0.167		130	186	0.34	1.7	23.0	0.18
	6 weeks	36.9	36.9	23.8	81.0	93.0	0.263	0.276	0.523	134	193	0.60	4.8	51.7	0.39
	3 months	40.5	39.7		109.0		0.321	0.264		140	226	0.78		73.5	0.53
3	Control	45.7	40.1	23.8	169.0		0.227	0.204		199	325	0.85	15.8	129.0	0.65
	5 days	14.6	8.1	6.4	14.4		0.057	0.078		180	300	0.08	−2.5	1.6	0.01
	10 days	15.3	10.6	7.6	66.0		0.122	0.070		140	228	0.47	2.7	52.6	0.38
	1 month	29.0	27.3	18.6	130.0		0.224			132	200	0.98	13.9	104.0	0.79
4	Control	38.8	40.4	26.6	105.0	124.0	0.263		0.681	135	213	0.78	7.7	74.0	0.55
	5 days	13.6	11.0	9.4	9.7	10.9	0.095	0.087	0.040	153	213	0.07	−0.4	−2.6	−0.02

Diodrast clearances were not done in the first two experiments or at low plasma levels in the third experiment with dog 1.

phate. Free diodrast (not bound to albumin) was determined from a nomogram constructed from the data of Smith and Smith (1938).

RESULTS. The determined clearances and blood flows and calculations based on these data are summarized in table 1. Each control represents the average of 6–8 clearance periods in two experiments; the data following uranium poisoning are averages of 3–4 clearance periods in single experiments.

The plasma flow (column 11) is the average obtained from creatinine and inulin plasma clearances and extractions and from whole blood diodrast data and hematocrit values except in a few instances where one method gave a value inconsistent with the other two.

Column 13 shows the extent to which C_D approximates the independently measured plasma flow. This value falls from an average of 81 per cent in the control periods to 7 per cent following uranium.

Despite the fact that C_I is probably no longer an accurate measure of glomerular filtration because of back diffusion of inulin in the damaged tubules, it may nevertheless be used as a minimum measure of this function. On this basis the "tubular D plasma clearance" as defined by White (1940b) may be calculated and the ratio of this value to RPF taken as the "tubular extraction", a measure of the fraction of RPF cleared of D by tubular activity. Tubular D clearance (column 15), following uranium administration, falls to practically zero in one case and to a negative value in the other two, evidence of complete suppression of tubular D secretion. The tubular extraction (column 16) falls from an average of 0.58 to a negative value. The effect of uranium on the maximum rate of tubular excretion of D (Tm) is shown in column 14.

In charts 1 and 2 the output of diodrast per minute and the plasma diodrast clearance respectively are plotted against the plasma diodrast level using the data obtained in the individual clearance periods both before and five days after uranium poisoning.

DISCUSSION. The changes in C_I and C_D following uranium poisoning are consistent with those previously reported (Hayman et al., 1939). Both are reduced, the latter to a greater extent. The urea clearance is also reduced and approximately parallels C_I. The diodrast clearance, however, is reduced to a much greater extent in all cases falling below C_I (columns 3–7).

Coincident with the reduction in clearances is a parallel reduction in extractions (columns 8–10) so that, in the acute stage of uranium poisoning, there is no significant change in RPF (column 11). In two of the animals there was a subsequent slight, possibly permanent, lowering of RPF.

The principal effect of uranium poisoning observed histologically is tubular damage.[3] In the absence of evidence of marked glomerular change, it seems reasonable to explain the reduction in C_I and C_C on the basis of extensive tubular back diffusion of these substances. The behavior of C_D further strengthens this

[3] Dog 4 was sacrificed six days after poisoning and kidney sections were examined histologically by Dr. H. Goldblatt. The findings were similar to those described by MacNider (1924) and summarized briefly by Hayman et al. (1939), namely, marked tubular necrosis with very slight glomerular involvement.

hypothesis. The tubular component of D extraction is eliminated in the acute state of poisoning when the most marked tubular damage is apparent. Diodrast clearance is reduced to the level of C_I and C_C and D is thus being removed from the blood by the same mechanism as are I and C. Actually C_D, like C_C, is lower than C_I presumably indicating that not only has D secretion been abolished but also that back diffusion of a portion of the glomerular output has occurred. Since both C and D (by comparison with I) apparently diffuse back into the circulation across the damaged tubular membrane, it is quite possible that the reduction of C_I as well as of C_C and C_D may be largely due to this process rather than to glomerular damage.

The reduction of tubular D plasma clearance and tubular D extraction to zero or negative values is a more striking presentation of the fact that tubular secretion of D is absent and may even be replaced to some extent by back diffusion of D. The fact that Tm, a measure of the rate of tubular excretory function independent of glomerular activity, falls to a negative value after poisoning can only be interpreted as additional evidence of complete replacement of tubular secretion by some tubular back diffusion.

The use of C_D as a measure of effective RPF is dependent on the ability of the tubule cells to remove essentially all of the D presented to them by the blood. The complete loss of this function resulting from uranium poisoning invalidates the use of C_D as an index of RPF and leads to impossibly high values for the filtration fraction (C_I/C_D). This suggests that any observed high value of the filtration fraction should be interpreted with the possibility in mind that it may be indicative of tubular dysfunction and may not necessarily be due to constriction of the efferent glomerular arterioles.

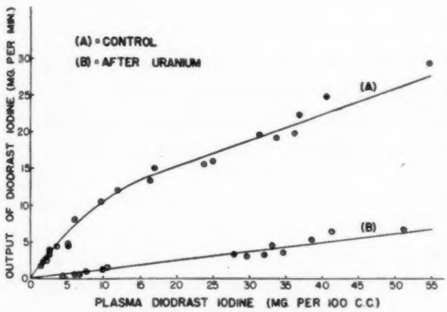

Chart 1

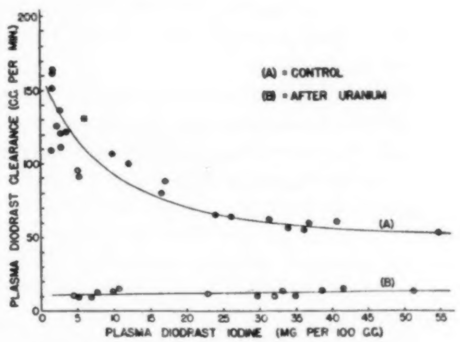

Chart 2

Chart 1. The relation of diodrast iodine output to plasma level before and after uranium poisoning.

Chart 2. The relation of plasma diodrast clearance to plasma diodrast iodine level before and after uranium poisoning.

In the control curve of chart 1 the output of D increases rapidly with increasing plasma level until at about 15 mgm./100 cc., the tubular capacity for D excretion

is saturated. Beyond this level the output increases linearly with plasma level and reflects only an increase in the amount filtered by the glomeruli. After uranium, the output is a linear function of plasma level with no initial rapid rise at low levels since only glomerular activity is involved. The slope of this line is less than the slope of the straight line portion of the control curve due to the apparent lowering of the level of glomerular filtration resulting from back diffusion in the damaged tubules.

As shown in chart 2, C_D in the normal kidney steadily decreases as the plasma D level is raised and the tubular mechanism for excretion becomes saturated, but following uranium poisoning tubular secretion of D is abolished and this relationship does not appear. Diodrast clearance following poisoning is low even at low plasma levels due to back diffusion of D from the glomerular filtrate but it is not further depressed as plasma levels are raised which is in sharp contrast to the control data.

As recovery from poisoning takes place, all clearances return toward normal values. The rate of improvement in the early stages is greatest in the case of C_D. Improvement in kidney function, as shown by the increase in C_I and C_C as well as by the return of concentrating ability and the disappearance of albuminuria and glycosuria which is observed in these dogs, coincides with recovery from tubular damage as indicated by the return of C_D toward normal levels.

CONCLUSIONS

1. The injury to the dog kidney produced by uranium causes a decrease in all clearances but negligible changes in the renal plasma flow. Diodrast plasma clearance is reduced to the level of inulin clearance or lower.

2. The reduction of diodrast plasma clearance to the level of apparent glomerular filtration is the result of the complete loss of the ability of the tubules to secrete diodrast.

3. The reduction in glomerular filtration is probably due in large part to back diffusion in the damaged tubules rather than to glomerular damage per se.

4. The use of diodrast plasma clearance as a measure of renal plasma flow and of inulin clearance as a measure of glomerular filtration is not justified in the uranium damaged kidney.

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INTRAMUSCULAR PRESSURE DURING LIFE AND AFTER DEATH[1]

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Henderson's concept of the existence of a venopressor mechanism postulates that venous pressure and venous flow are dependent on muscle tonus. When muscle tonus fails, venous pressure and venous flow must fail; thus, in shock, the tonus of the muscles lessens and collapse of the peripheral circulation ensues. Shock according to Henderson's concept is "hypotonia" (1).

In 1936 Henderson (2) devised an instument for the measurement of intramuscular pressure. A modification of it as proposed in 1936 by Kerr and Scott (3) was accepted by Henderson (1). Small quantities of 0.8 per cent saline were injected into the muscle mass under measurable increments of pressure. The muscle accepts such injections at reproducible pressures which in the normal human adult varies from 60–90 mm. H_2O. The increment of pressure, resulting from the ability of the muscle to accept injections of saline at reproducible levels, Henderson termed intramuscular pressure (2). A considerable literature has demonstrated the validity of the measurement of intratissue pressure by forcing minute amounts of saline into the tissue (3, 4, 5). These pressures vary significantly with the physiologic and pathologic states of the muscular and circulatory systems. Henderson has shown that intramuscular pressure falls to low levels after anesthesia and surgical procedures (6) and should be low in shock (1). We have verified and extended his observations in postoperative depression and in shock-like states associated with hemorrhage, surgical procedures under anesthesia, and in acute infections (7, 8, 9).

In Henderson's concept, intramuscular pressure is a measurement of muscle tonus, and muscle tonus is measurable during life as intramuscular pressure. Henderson used the term tonus according to the definition of Johannes Müller (19); *as a slight contractile tension characteristic of normal skeletal muscle when at rest.* This, Müller attributed to the influence continually exerted upon the muscle by the nerve centers in the brain. Such rhythmic activity of discrete muscle bundles within the muscle mass should be demonstrable by the detection of electrical action currents. Dr. L. F. Nims in the neuro-anatomy laboratory at Yale University, using a cathode ray oscillograph, demonstrated the presence of rhythmic action currents in the resting biceps brachii muscle of the human being. Thus Müller's contractile tension in the muscle at rest is demonstrated to be related to activity of discrete muscle bundles within the muscle mass. As Henderson and others have shown (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 15) this

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tension is measurable at reproducible increments by the injection of minute amounts of saline into the muscle, as intramuscular pressure. It increases as the muscle performs a definite amount of work, and decreases to a definite resting level as the muscle mass relaxes (11). The measurement of intratissue pressure in the muscle during life is a measurement of muscle tonus.

Krogh (13) showed that individual muscle fiber groups are under different states of contraction and relaxation. The amount of blood filling the capillary and venule bed is related to the degree of activity of the muscle, and to the number of muscle bundles at any one moment that are in contraction (1, 11, 13). During maximum contraction the capillary and venule bed of the muscle is gorged with blood. Such increasing degrees of muscle activity should show summation of the electrical action currents, that have been demonstrated in the muscle at rest. This phenomenon, too, Henderson demonstrated in his laboratory. He showed that increasing amounts of summation of electrical activity occurred with increasing degrees of work, performed by the biceps brachii in human beings. The increasing degrees of summation of the action currents with work is correlated with increasing levels of intramuscular pressure. Decreasing activity as the muscle relaxes is similarly paralleled by a decrease in the increments of intratissue pressure to the resting level (11).

Krogh's morphologic description of the increasing and decreasing vascularity within a muscle, related to the degree of activity, and the number of muscle bundles contracting within the muscle mass, is in reality the description of a microscopic venous pump (1, 11).

The evidence at hand now lends strong support for Henderson's postulate of the existence of a venous pump in the circulation of the human being. This pump is the mass of skeletal muscle, including the diaphragm. The energy for the pump is derived from the isometric tonic activity of the discrete muscle bundles when the muscle is at rest, and by active contraction of muscles, in the performance of work.

The energy of tonic muscle activity as well as that of the contracting muscle is transferred to the venous column and is reflected in the volume of venous flow to the heart, and in the effective level of venous pressure.

When muscle tonus, i.e., intramuscular pressure, fails, the effective venous pressure and venous flow necessary to maintain ventricular filling decline, cardiac output (volume of flow) decreases, and failure of the peripheral circulation ensues. This is the venopressor mechanism.

We too have found that the muscle accepts injections of saline at reproducible increments which range in the normal human between 60 and 90 mm. H_2O (7, 14) and vary significantly in shock and in shock-like states (7, 8, 9). It is evident from our studies that the energy of the tonic tension of muscle fibers, measured in the muscle mass as intramuscular pressure, can be transferred to the venous bed and be manifested as venous flow and alterations in venous pressure (7, 8, 9, 14, 15). Contrariwise can the pressures within the venules which so richly surround muscle fibers be imparted to the mass of muscle as increments of intratissue pressure? Is the resistance of the muscle mass to the injection of fluid,

viz., intramuscular pressure, a result of the pressure within the venous bed measured as venous pressure?

Methods for the measurement of intramuscular and venous pressure. Over one thousand measurements of intramuscular pressure have been made in our various studies (7, 8, 9, 14, 15). Henderson's method (6) was used in about half of our observations, and the instrument devised by Gunther and Henstell (15) for the simultaneous and repeated determination of intramuscular and venous pressure in the remainder. Venous pressure was measured by the direct method when Henderson's instrument was used. The level of the auricle was taken at 5 cm. below the level of the chest in recumbency in both methods.

Methods of study. The tourniquet experiment described by Wells et al. (4) was employed, using the biceps brachii muscle. Venous pressure was measured in the ante-cubital vein. Simultaneous measurements of intramuscular and venous pressure were made at different increments of venous pressure up to 58 cm. H_2O.

Bedside observations were made on patients suffering from congestive (right heart) failure wherein venous pressures up to 30 cm. H_2O were encountered. Measurements were taken simultaneously in the ante-cubital vein and in the biceps brachii muscle.

Bedside studies were conducted in three patients suffering from syphilis with tabes dorsalis. Simultaneous measurements were made in the antecubital vein and in the biceps brachii muscle in the upper extremity and in the long saphenous vein and the gastrocnemius muscle of the lower extremity. The tourniquet experiment was repeated in the arm and in the leg at different increments of venous pressure.

Studies were made in the operating room during the course of anesthesia and surgical procedures in which the Trendelenburg position was used during surgery. Intramuscular pressure was measured in the biceps brachii and venous pressure in the antecubital vein. The level of the auricles was taken at chest level during the Trendelenburg position.

Observations were made in human beings and in the frog (Rana pipiens) with the needle in situ, immediately before and after death. In human beings the biceps brachii and antecubital veins were used; in the frog, intramuscular pressure was measured in the gastrocnemius and the adductor magnus muscles of the thigh.

These pressures were studied in the frog before and immediately after clamping the branches of the truncus arteriosus at the base of the heart, and of the dorsal aorta caudal to the iliac arteries, to determine the effect of the loss of venous pressure on intramuscular pressure.

Results of experiments. The effect of artificially induced high increments of venous pressure on intramuscular pressure was studied in 4 tourniquet experiments. The venous pressures induced up to 58 cm. H_2O maintained for 38 minutes showed no significant effect on intramuscular pressure of the corresponding biceps brachii muscle (fig. 1 a and 1 b). One tourniquet experiment was made in the arm and the leg of a patient with tabes dorsalis. He had low

initial values for both the venous and intramuscular pressure. Venous pressure was 3.6 cm. H_2O in the antecubital vein and 4.0 cm. in the biceps brachii, and 40 mm. H_2O in the gastrocneumius muscle. Increasing the levels of venous pressure to 36 cm. H_2O in the antecubital vein and 19 cm. H_2O in the long saphenous vein did not alter the intramuscular pressure in either the biceps or the gastrocnemius (fig. 2).

Four patients suffering from congestive heart failure were studied. They showed venous pressures from 18 to 30 cm. H_2O. The intramuscular pressure

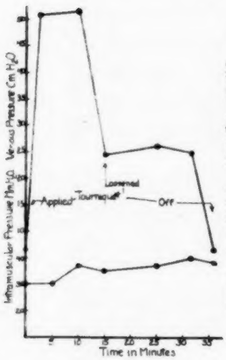

Fig. 1a

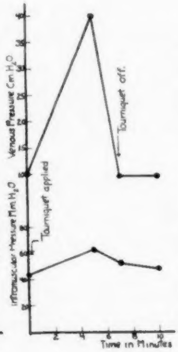

Fig. 1b

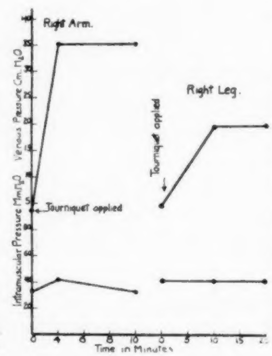

Fig. 2

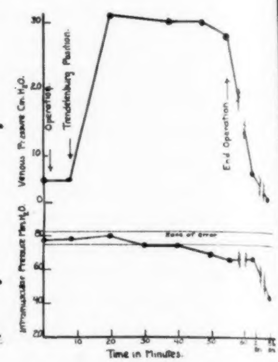

Fig. 3

Fig. 1a, 1b. Showing the effect of high levels of venous pressure on intramuscular pressure in the biceps brachii in the human.

Fig. 2. Showing the effect of high levels of venous pressure on the intramuscular pressure in the biceps brachii and the gastrocnemius muscles in the human, suffering from syphilis with tabes dorsalis.

The initial values for intramuscular and venous pressures are below the normal range. The patient did not show signs of peripheral circulatory inadequacy.

Fig. 3. Showing the effect of high levels of venous pressure, induced by gravity, in the Trendelenburg position, during the course of operation under anesthesia.

The high increments of venous pressure did not affect the preoperative level of intramuscular pressure. The usual drop in intramuscular pressure occurred after 40 minutes of surgery despite the maintained high venous pressure. This drop preceded the fall in venous pressure which occurred later. The entire course of events is identical to those which occur in other patients during anesthesia and operation, in normal recumbency and in whom venous pressures were normal at the outset and during the initial period of operation.

values were well within the normal range of 60–90 mm. H_2O observed in persons without congestive heart failure (table 1).

Two patients undergoing pelvic operations in the Trendelenburg position were observed throughout the course of surgery. When placed in this position, the head is considerably lower than the feet and the forces of gravity greatly increase the physiological values of the venous pressure. Venous pressure to 27 and 31 cm. H_2O plus or minus 3 mm. H_2O were recorded. No significant alterations of intramuscular pressure were observed before or after the venous pressure was

increased to these high levels by the effect of gravity (fig. 3 a, 3 b). The Trendelenburg position and the high increment of venous pressure was maintained for from 40–50 minutes during the operation. The high level of the venous pressures in the Trendelenburg position did not prevent the usually observed fall of

TABLE 1

Intramuscular and venous pressures in congestive heart failure

PATIENT	TIME	INTRA-MUSCULAR PRESSURE	VENOUS PRESSURE	BLOOD PRESSURE	REMARKS
		mm. H_2O	*cm. H_2O*	*mm. Hg*	
A10		66	20		
A10a		83	18		Rheumatic heart disease. Auricular fibrillation. Ether time 37 seconds. Vital capacity 1500 cc.
A10b		55	20		Pericardial effusion
A41		64	25		Pericardial effusion
		65	26		
		68	30		Patient excited. Pericardial thoracentesis was attempted
A7		95	11		Before anesthesia
	10 min.	95	11		Before surgery and after inhalational anesthesia
	40 min.	85	27		Trendelenburg position; 40 min. after onset of anesthesia and 30 min. after onset of surgery. Intramuscular pressure still within range of error of the method, but falling
	120 min.	38	4		Later it reached a low level
B7		78	8.0	140/86	Before surgery and anesthesia
	9 min.	80	8.0		Surgery
	19 min.	83	31.0	160/80	Trendelenburg position
	34 min.		31 ± 3		Rapid respiratory fluctuation
	37 min.	75	28		
	40 min.	76	30	170/80	
	48 min.	68	28		
	58 min.	66	28	160/80	Operation ended
	8 hrs. and 43 min.	43	3.6		Post-operative depression

intramuscular pressure which likewise occurs after 40–50 minutes of continuous anesthesia and surgery in the ordinary recumbent position. As has been previously observed, the fall in intramuscular pressure preceded the drop in venous pressure. This phenomenon was recorded while the venous pressure was still at a very high level.

Clamping the branches of the truncus arteriosus at the base of the heart of the frog (Rana pipiens) was followed by a cessation of the heart beat which returned after the clamp was removed. With the arterial outflow clamped and the heart at rest, no significant alterations in intramuscular pressure occurred in the gastrocnemius or adductor magnus muscles (table 2). There was little change at 11 hours, but at 24 hours the intramuscular pressure had risen to double the previous value (table 2).

TABLE 2

Showing the effect on intramuscular pressure in the gastrocnemius of the frog (Rana pipiens) of: 1. Loss of venous pressure by blocking the common iliac artery. 2. Loss of venous pressure by blocking the outflow of the heart. 3. Effect of death, viz., cessation of the heart beat, immediately, 11 hours later and 24 hours later. 4. Effect of death in the human, immediately after shock and peripheral circulatory collapse (loss of venous pressure). 5. Effect of death, 24 hours later, in the human (loss of venous pressure)

	INTRAMUSCULAR PRESSURE	REMARKS
	mm. H_2O	
R. P.*	36	Before and after pithing
	33	Dorsal aorta clamped caudal to the iliacs
	28	Considerable hemorrhage. Before
	26	Branches of truncus arteriosus clamped. Heart stopped beating
	26	Clamps released. Heart beating
	26	Branches of truncus arteriosus clamped. Heart stopped beating
	26	Apparent death. Heart not beating
	29	Heart stopped beating 11 hours
	66	24 hours after heart ceased to beat
A57†	25	5 minutes before death, patient in shock and peripheral circulatory collapse
	25	15 minutes after death. No heart beat
M. C.†	60	Before death. Congestive heart failure
	125	24 hours after death

* Gastrocnemius muscle used in the frog (Rana pipiens).
† Biceps brachii muscle used in the human.

Immediately after death in the human being, a cessation of the heart beat in the frog, the intramuscular pressure did not drop. The levels immediately before and immediately after death were identical (table 2).

The lowest levels to which intramuscular pressure fell before death in the frog or in the human patient was 18 mm. H_2O (10).

Discussion. Our findings verified those of Wells et al. (4) who also were unable to change the level of intramuscular pressure in the biceps brachii by raising venous pressure to high levels with a tourniquet. However, when the tourniquet was placed on the leg and the intramuscular pressure measured in the soleus and

anterior tibial muscles, they found that venous pressures above 20 cm. H_2O increased decidedly the intramuscular pressure in these muscles.

The difference in results obtained in the same experiment on the biceps brachii in the arm, and the soleus and anterior tibial muscles in the leg are explainable on an anatomical basis. The latter muscles possess a very tight fascial covering. The biceps brachii and the gastrocnemius muscles on the other hand do not possess a tight fascia. Thus, beyond certain increments of pressure, the dynamics of a closed, relatively inelastic space, comes into play in muscles covered by tight fascia. The fascia of these anti-gravity muscles serve a useful purpose in the support of the long column of venous blood in the extremities in erect posture (4).

It is an interesting coincidence that up to 20 mm. H_2O venous pressure, there was no significant change in intramuscular pressure even in these tightly covered muscles. Twenty centimeters of water venous pressure is commonly encountered clinically in congestive heart failure. We report herein observations on patients with heart failure with venous pressures up to 30 cm. H_2O without significant alterations in intramuscular pressure (table 1). We feel that muscles that do not possess a tightly investing fascia, give a more accurate reflection of intratissue pressure, in relation to the venules of the venous bed which encompass its fibers, in physiologic and pathologic states, than do the muscles with a tight fascia whose intratissue pressure is subject to the dynamics of increments within a closed space.

We have shown that the energy of the muscles, measured as intramuscular pressure, is transferable to the venous bed in terms of an increase in venous pressure (8, 9, 14). Contrariwise, it is apparent from these experiments in which venous pressure was raised to high levels through the use of the tourniquet, through gravity, and with venoconstrictor drugs (9), that the energy of the venous bed, expressed as an increased level of venous pressure, is not transferable to the muscle mass in terms of intramuscular pressure. In none of these experiments did the intramuscular pressure significantly change in the face of high levels of venous pressure. Even more significant, in our opinion, was the fall in intramuscular pressure that occurred after 40–50 minutes of anesthesia and surgery, in the presence of the high level of venous pressure, which had been maintained for 38 minutes through the forces of gravity. The drop in intramuscular pressure in the presence of a high venous pressure was in no way different from that previously reported by the authors (14) after 40–50 minutes of anesthesia and surgery in the recumbent position, wherein the venous pressure was within normal limits at the beginning of the operation. In a similar fashion, in both circumstances, the initial decrease in intramuscular pressure preceded the drop in venous pressure by 40–50 minutes.

Sympathomimetic drugs which have a venoconstrictor action as well as a pressor action increase venous pressure for from 20 to 40 minutes, but in no way change the level of intramuscular pressure (14, 17). We have indicated that a compensatory mechanism exists akin to the vasoconstriction which increases peripheral resistance to maintain systolic blood pressure which through venoconstriction, alone, sustains venous pressure during surgical procedures for pe-

riods of from 20 to 40 minutes after intramuscular pressure has failed (9, 14). However, if the level of intramuscular pressure continues to remain low beyond this period of time the mechanism fails and venous pressure declines (7, 8, 9, 10, 14).

A total loss of venous pressure, such as occurs after death in the patient and after clamping of the arterial outflow from the heart of the frog, with stoppage of the heart, did not alter the level of intramuscular pressure that existed before the clamping experiment, and before death in the patient or for eleven hours after cessation of the heart beat in the frog (table 2).

We did not encounter intramuscular pressures below 18 mm. H_2O immediately after death in any patient (10) or in the frog. This is the lowest level to which intramuscular pressure falls in postoperative depression, in shock, and in shock-like states in the human being.

Eighteen millimeter H_2O pressure must be the resiliance pressure of the mass of the muscle itself, for as rigor mortis appears the resistance of the muscle mass to the injection of fluid becomes very high. Intramuscular pressures above 18 mm. H_2O during life probably represent the energy of the muscles that is effective in the dynamics of peripheral circulation.

The evidence is strong that intramuscular pressure is a measurement of a quality of the living muscle called tonus. But at death the value of this measurement ceases, inasmuch as tonus disappears with death (2), but the resistance of the muscle to injection of fluid does not disappear with death.

Clinicians have long been aware of the relaxed state of the muscles of a patient suffering from syphilis with tabes dorsalis. Physiologists explained these phenomena in terms of loss of muscle tonus or as hypotonia (16). We have observed low values for intramuscular pressure in tabetic patients. As shown in figure 1, in such a patient, not only was the intramuscular pressure in the biceps brachii and the gastrocnemius muscles lower than is commonly found in the normal healthy individual, but the venous pressure measured in the antecubital vein was likewise lower than was normally found.

We have never observed a low venous pressure with a normal intramuscular pressure nor have we ever observed a low venous pressure without a low level for intramuscular pressure. We have followed patients before and during the development of postoperative depression and shock and into shock-like states with peripheral circulatory collapse and have never observed venous pressure to fall first, and intramuscular pressure later. Contrariwise, after the first 40–50 minutes of anesthesia and surgery, intramuscular pressure fails before the venous pressure drops. Thus, after intramuscular pressure had been depressed for a period upward of 40 minutes, the venous pressure always declined. When a patient was seen for the first time and found to be in shock with peripheral circulatory failure, an initial low intramuscular pressure was always accompanied by a low venous pressure. However, when we had the opportunity to study a patient during the development of shock and peripheral collapse, we found that intramuscular pressure failed first, and the venous pressure later.

The low level of intramuscular pressure seen in peripheral collapse, can be restored to the normal level by the intravenous administration of 10 cc. of a 20

per cent solution of pyridine-b-carboxylic-acid diethylamide[5]. Such a restoration is always accompanied by a simultaneous increase in venous pressure. Similarly, after the inhalation of carbon dioxide diluted in air in the normal (18) and after over-ventilation tetany in the normal, increases in intramuscular and venous pressures were always observed to occur simultaneously (14).

In our experiments and observations, all that could be noted was *a*, increasing venous pressure by means of a tourniquet, by gravity or by sympathomimetic drugs did not increase intramuscular pressure; *b*, a high level of venous pressure did not prevent the fall of intramuscular pressure (after 40–50 min. of surgery and anesthesia) which in turn preceded the fall of venous pressure; *c*, in the hypotonia of tabes dorsalis both intramuscular and venous pressures were found to be at low levels; *d*, a simultaneous increase of intramuscular and venous pressures always occurred after any agent that increased intramuscular pressure. When clinical improvement in the peripheral circulation occurred with a rise in venous pressure, evidence could not be demonstrated that intramuscular pressure increased before the venous pressure, analogous to the observation that the muscular phenomenon preceded the venous phenomenon when failure in the peripheral circulation occurred.

SUMMARY. Increasing the venous pressure to high increments by use of the tourniquet did not increase the level of intramuscular pressure in the human being.

Increasing the level of venous pressure by gravity in the Trendelenburg position for periods up to 50 minutes did not increase intramuscular pressure in the patient, nor did the maintained high level of venous pressure prevent a fall of intramuscular pressure which occurred after 40–50 minutes of surgical procedure under anesthesia, which in turn preceded the fall in venous pressure.

Sudden clamping of the arterial circulation and at the truncus arteriosus and in the dorsal aorta in the frog did not lower intramuscular pressure in the gastrocnemius or adductor magnus muscles.

A loss of venous pressure after death in the human patient, and after cessation of the heart beat in the frog did not immediately alter the previously existing level of intramuscular pressure, nor did it change in the frog 11 hours after cessation of the heart beat.

Eighteen millimeters H_2O is the lowest level of intramuscular pressure obtained in human beings or in the frog immediately after death; this is the level to which intramuscular pressure falls before death in the shock state in patients, when peripheral collapse has ensued.

The resistance of the muscle mass to the injection of fluid (intramuscular pressure) increases after death and becomes high with the appearance of rigor mortis.

CONCLUSIONS

1. The resistance of the muscle mass to injections of saline at reproducible levels is a valid measurement of tissue pressure but only during life is it a measurement of the property called tonus.

[5] Coramine brand of Nikethamide, Ciba.

2. Intramuscular pressure increments above 18 mm. H_2O represent intratissue energy that is transferable to the venous bed. This transfer of energy can be measured in terms of increments of venous pressure.

3. Venous pressure on the other hand is not a determinant of intramuscular pressure, and the energy of the venous bed, measured as venous pressure, is not transferable to the muscle mass, measured as intramuscular pressure.

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